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RETROVIRAL VECTORS COMPRISING A FUNCTIONAL SPLICE DONOR SITE AND A FUNCTIONAL
SPLICE ACCEPTOR SITE

The present invention relates to a vector.

- 5 In particular, the present invention relates to a novel system for packaging and expressing genetic material in a retroviral particle.

More in particular, the present invention relates to a novel system capable of expressing a retroviral particle that is capable of delivering a nucleotide sequence of interest
10 (hereinafter abbreviated as "NOI") - or even a plurality of NOIs - to one or more target sites.

In addition, the present invention relates to *inter alia* a novel retroviral vector useful in gene therapy.

15 *BACKGROUND OF THE INVENTION*

Gene therapy may include any one or more of: the addition, the replacement, the deletion, the supplementation, the manipulation etc. of one or more nucleotide sequences in, for example, one or more targeted sites - such as targeted cells. If the targeted sites are targeted cells, then the cells may be part of a tissue or an organ.

- 20 General teachings on gene therapy may be found in Molecular Biology (Ed Robert Meyers, Pub VCH, such as pages 556-558).

By way of further example, gene therapy can also provide a means by which any one or more of: a nucleotide sequence, such as a gene, can be applied to replace or supplement
25 a defective gene; a pathogenic nucleotide sequence, such as a gene, or expression product thereof can be eliminated; a nucleotide sequence, such as a gene, or expression product thereof, can be added or introduced in order, for example, to create a more favourable phenotype; a nucleotide sequence, such as a gene, or expression product thereof can be added or introduced, for example, for selection purposes (i.e. to select
30 transformed cells and the like over non-transformed cells); cells can be manipulated at the molecular level to treat, cure or prevent disease conditions - such as cancer (Schmidt-

Wolf and Schmidt-Wolf, 1994, *Annals of Hematology* 69;273-279) or other disease conditions, such as immune, cardiovascular, neurological, inflammatory or infectious disorders; antigens can be manipulated and/or introduced to elicit an immune response, such as genetic vaccination.

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In recent years, retroviruses have been proposed for use in gene therapy. Essentially, retroviruses are RNA viruses with a life cycle different to that of lytic viruses. In this regard, a retrovirus is an infectious entity that replicates through a DNA intermediate. When a retrovirus infects a cell, its genome is converted to a DNA form by a reverse transcriptase enzyme. The DNA copy serves as a template for the production of new RNA genomes and virally encoded proteins necessary for the assembly of infectious viral particles.

There are many retroviruses and examples include: murine leukemia virus (MLV), human immunodeficiency virus (HIV), equine infectious anaemia virus (EIAV), mouse mammary tumour virus (MMTV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV), Moloney murine leukemia virus (Mo-MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney murine sarcoma virus (Mo-MSV), Abelson murine leukemia virus (A-MLV), Avian myelocytomatosis virus-29 (MC29), and Avian erythroblastosis virus (AEV).

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A detailed list of retroviruses may be found in Coffin *et al* ("Retroviruses" 1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763).

Essentially, all wild type retroviruses contain three major coding domains, *gag*, *pol*, *env*, which code for essential virion proteins. Nevertheless, retroviruses may be broadly

divided into two categories: namely, "simple" and "complex". These categories are distinguishable by the organisation of their genomes. Simple retroviruses usually carry only elementary information. In contrast, complex retroviruses also code for additional regulatory proteins derived from multiple spliced messages.

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Retroviruses may even be further divided into seven groups. Five of these groups represent retroviruses with oncogenic potential. The remaining two groups are the lentiviruses and the spumaviruses. A review of these retroviruses is presented in "Retroviruses" (1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 1-25).

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All oncogenic members except the human T-cell leukemia virus-bovine leukemia virus group (HTLV-BLV) are simple retroviruses. HTLV, BLV and the lentiviruses and spumaviruses are complex. Some of the best studied oncogenic retroviruses are Rous sarcoma virus (RSV), mouse mammary tumour virus (MMTV) and murine leukemia virus (MLV) and the human T-cell leukemia virus (HTLV).

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The lentivirus group can be split even further into "primate" and "non-primate". Examples of primate lentiviruses include the human immunodeficiency virus (HIV), the causative agent of human auto-immunodeficiency syndrome (AIDS), and the simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the prototype "slow virus" visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV).

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A distinction between the lentivirus family and other types of retroviruses is that lentiviruses have the capability to infect both dividing and non-dividing cells (Lewis *et al* 1992 EMBO. J 11: 3053-3058; Lewis and Emerman 1994 J. Virol. 68: 510-516). In contrast, other retroviruses - such as MLV - are unable to infect non-dividing cells such as those that make up, for example, muscle, brain, lung and liver tissue.

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During the process of infection, a retrovirus initially attaches to a specific cell surface receptor. On entry into the susceptible host cell, the retroviral RNA genome is then copied to DNA by the virally encoded reverse transcriptase which is carried inside the parent virus. This DNA is transported to the host cell nucleus where it subsequently integrates into the host genome. At this stage, it is typically referred to as the provirus. The provirus is stable in the host chromosome during cell division and is transcribed like other cellular proteins. The provirus encodes the proteins and packaging machinery required to make more virus, which can leave the cell by a process sometimes called "budding".

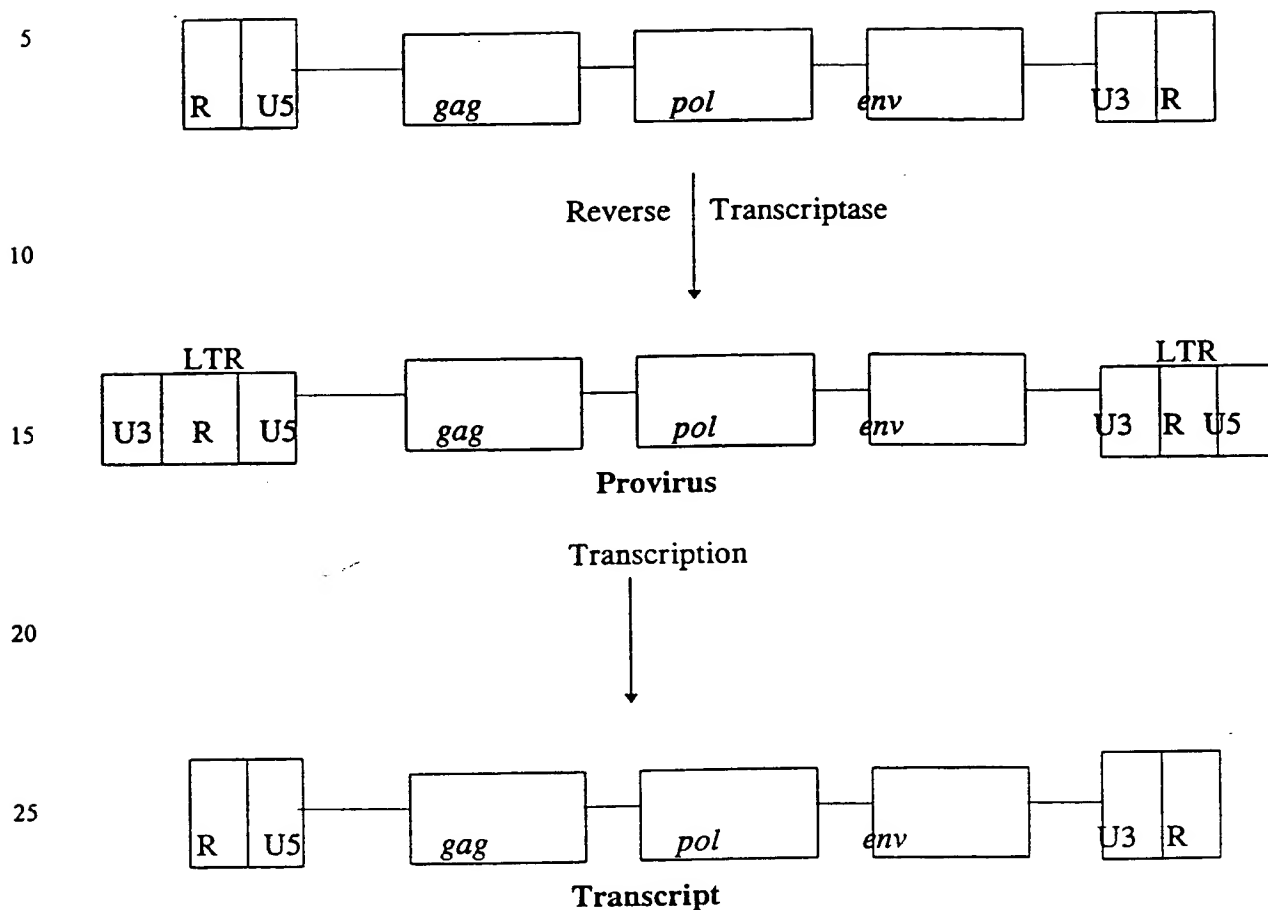
As already indicated, each retroviral genome comprises genes called *gag*, *pol* and *env* which code for virion proteins and enzymes. In the provirus, these genes are flanked at both ends by regions called long terminal repeats (LTRs). The LTRs are responsible for proviral integration, and transcription. They also serve as enhancer-promoter sequences. In other words, the LTRs can control the expression of the viral gene. Encapsidation of the retroviral RNAs occurs by virtue of a *psi* sequence located at the 5' end of the viral genome.

The LTRs themselves are identical sequences that can be divided into three elements, which are called U3, R and U5. U3 is derived from the sequence unique to the 3' end of the RNA. R is derived from a sequence repeated at both ends of the RNA and U5 is derived from the sequence unique to the 5' end of the RNA. The sizes of the three elements can vary considerably among different retroviruses.

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For ease of understanding, simple, generic structures (not to scale) of the RNA and the DNA forms of the retroviral genome are presented below in which the elementary features of the LTRs and the relative positioning of *gag*, *pol* and *env* are indicated.

Virion RNA



As shown in the diagram above, the basic molecular organisation of an infectious retroviral RNA genome is (5') R - U5 - *gag*, *pol*, *env* - U3-R (3'). In a defective retroviral vector genome *gag*, *pol* and *env* may be absent or not functional. The R regions at both ends of the RNA are repeated sequences. U5 and U3 represent unique sequences at the 5' and 3' ends of the RNA genome respectively.

Reverse transcription of the virion RNA into double stranded DNA takes place in the cytoplasm and involves two jumps of the reverse transcriptase from the 5' terminus to the 3' terminus of the template molecule. The result of these jumps is a duplication of sequences located at the 5' and 3' ends of the virion RNA. These sequences then occur fused in tandem on both ends of the viral DNA, forming the long terminal repeats

(LTRs) which comprise R U5 and U3 regions. On completion of the reverse transcription, the viral DNA is translocated into the nucleus where the linear copy of the retroviral genome, called a preintegration complex (PIC), is randomly inserted into chromosomal DNA with the aid of the virion integrase to form a stable provirus. The number of possible sites of integration into the host cellular genome is very large and very widely distributed.

The control of proviral transcription remains largely with the noncoding sequences of the viral LTR. The site of transcription initiation is at the boundary between U3 and R in the left hand side LTR (as shown above) and the site of poly (A) addition (termination) is at the boundary between R and U5 in the right hand side LTR (as shown above). U3 contains most of the transcriptional control elements of the provirus, which include the promoter and multiple enhancer sequences responsive to cellular and in some cases, viral transcriptional activator proteins. Some retroviruses have any one or more of the following genes such as *tat*, *rev*, *tax* and *rex* that code for proteins that are involved in the regulation of gene expression.

Transcription of proviral DNA recreates the full length viral RNA genomic and subgenomic-sized RNA molecules that are generated by RNA processing. Typically, all RNA products serve as templates for the production of viral proteins. The expression of the RNA products is achieved by a combination of RNA transcript splicing and ribosomal framshifting during translation.

RNA splicing is the process by which intervening or "intronic" RNA sequences are removed and the remaining "exonic" sequences are ligated to provide continuous reading frames for translation. The primary transcript of retroviral DNA is modified in several ways and closely resembles a cellular mRNA. However, unlike most cellular mRNAs, in which all introns are efficiently spliced, newly synthesised retroviral RNA must be diverted into two populations. One population remains unspliced to serve as the genomic RNA and the other population is spliced to provide subgenomic RNA.

The full-length unspliced retroviral RNA transcripts serve two functions: (i) they encode the *gag* and *pol* gene products and (ii) they are packaged into progeny virion particles as genomic RNA. Sub-genomic-sized RNA molecules provide mRNA for the remainder of the viral gene products. All spliced retroviral transcripts bear the first exon, which spans the U5 region of the 5' LTR. The final exon always retains the U3 and R regions encoded by the 3' LTR although it varies in size. The composition of the remainder of the RNA structure depends on the number of splicing events and the choice of alternative splice sites.

10 In simple retroviruses, one population of newly synthesised retroviral RNA remains unspliced to serve as the genomic RNA and as mRNA for *gag* and *pol*. The other population is spliced, fusing the 5' portion of the genomic RNA to the downstream genes, most commonly *env*. Splicing is achieved with the use of a splice donor positioned upstream of *gag* and a splice acceptor near the 3' terminus of *pol*. The intron
15 between the splice donor and splice acceptor that is removed by splicing contains the *gag* and *pol* genes. This splicing event creates the mRNA for envelope (Env) protein. Typically the splice donor is upstream of *gag* but in some viruses, such as ASLV, the splice donor is positioned a few codons into the *gag* gene resulting in a primary Env translation product that includes a few amino-terminal amino acid residues in common
20 with Gag. The Env protein is synthesised on membrane-bound polyribosomes and transported by the cell's vesicular traffic to the plasma membrane, where it is incorporated into viral particles.

Complex retroviruses generate both singly and multiply spliced transcripts that encode
25 not only the *env* gene products but also the sets of regulatory and accessory proteins unique to these viruses. Complex retroviruses such as the lentiviruses, and especially HIV, provide striking examples of the complexity of alternative splicing that can occur during retroviral infection. For example, it is now known that HIV-1 is capable of producing over 30 different mRNAs by sub-optimal splicing from primary genomic
30 transcripts. This selection process appears to be regulated as mutations that disrupt

competing splice acceptors can cause shifts in the splicing patterns and can affect viral infectivity (Purcell and Martin 1993 J Virol 67: 6365-6378).

The relative proportions of full-length RNA and subgenomic-sized RNAs vary in infected cells and modulation of the levels of these transcripts is a potential control step during retroviral gene expression. For retroviral gene expression, both unspliced and spliced RNAs must be transported to the cytoplasm and the proper ratio of spliced and unspliced RNA must be maintained for efficient retroviral gene expression. Different classes of retroviruses have evolved distinct solutions to this problem. The simple retroviruses, which use only full-length and singly spliced RNAs regulate the cytoplasmic ratios of these species either by the use of variably efficient splice sites or by the incorporation of several *cis*-acting elements, that have been only partially defined, into their genome. The complex retroviruses, which direct the synthesis of both singly and multiply spliced RNA, regulate the transport and possibly splicing of the different genomic and subgenomic-sized RNA species through the interaction of sequences on the RNA with the protein product of one of the accessory genes, such as *rev* in HIV-1 and *rex* in HTLV-1.

With regard to the structural genes *gag*, *pol* and *env* themselves and in slightly more detail, *gag* encodes the internal structural protein of the virus. Gag is proteolytically processed into the mature proteins MA (matrix), CA (capsid) and NC (nucleocapsid). The *pol* gene encodes the reverse transcriptase (RT), which contains both DNA polymerase, and associated RNase H activities and integrase (IN), which mediates replication of the genome. The *env* gene encodes the surface (SU) glycoprotein and the transmembrane (TM) protein of the virion, which form a complex that interacts specifically with cellular receptor proteins. This interaction leads ultimately to fusion of the viral membrane with the cell membrane.

The Env protein is a viral protein which coats the viral particle and plays an essential role in permitting viral entry into a target cell. The envelope glycoprotein complex of retroviruses includes two polypeptides: an external, glycosylated hydrophilic polypeptide

(SU) and a membrane-spanning protein (TM). Together, these form an oligomeric "knob" or "knobbed spike" on the surface of a virion. Both polypeptides are encoded by the *env* gene and are synthesised in the form of a polyprotein precursor that is proteolytically cleaved during its transport to the cell surface. Although uncleaved Env proteins are able to bind to the receptor, the cleavage event itself is necessary to activate the fusion potential of the protein, which is necessary for entry of the virus into the host cell. Typically, both SU and TM proteins are glycosylated at multiple sites. However, in some viruses, exemplified by MLV, TM is not glycosylated.

Although the SU and TM proteins are not always required for the assembly of enveloped virion particles as such, they play an essential role in the entry process. In this regard, the SU domain binds to a receptor molecule, often a specific receptor molecule, on the target cell. It is believed that this binding event activates the membrane fusion-inducing potential of the TM protein after which the viral and cell membranes fuse. In some viruses, notably MLV, a cleavage event, resulting in the removal of a short portion of the cytoplasmic tail of TM, is thought to play a key role in uncovering the full fusion activity of the protein (Brody *et al* 1994 J Virol 68: 4620-4627; Rein *et al* 1994 J Virol 68: 1773-1781). This cytoplasmic "tail", distal to the membrane-spanning segment of TM remains on the internal side of the viral membrane and it varies considerably in length in different retroviruses.

Thus, the specificity of the SU/receptor interaction can define the host range and tissue tropism of a retrovirus. In some cases, this specificity may restrict the transduction potential of a recombinant retroviral vector. Here, transduction includes a process of using a viral vector to deliver a non-viral gene to a target cell. For this reason, many gene therapy experiments have used MLV. A particular MLV that has an envelope protein called 4070A is known as an amphotropic virus, and this can also infect human cells because its envelope protein "docks" with a phosphate transport protein that is conserved between man and mouse. This transporter is ubiquitous and so these viruses are capable of infecting many cell types. In some cases however, it may be beneficial, especially from a safety point of view, to specifically target restricted cells. To this end, several groups have engineered a mouse ecotropic retrovirus, which unlike its

amphotropic relative normally only infects mouse cells, to specifically infect particular human cells. Replacement of a fragment of an Env protein with an erythropoietin segment produced a recombinant retrovirus which then binds specifically to human cells that express the erythropoietin receptor on their surface, such as red blood cell precursors (Maulik and Patel 1997 "Molecular Biotechnology: Therapeutic Applications and Strategies" 1997 Wiley-Liss Inc. pp 45).

In addition to *gag*, *pol* and *env*, the complex retroviruses also contain "accessory" genes which code for accessory or auxillary proteins. Accessory or auxillary proteins are defined as those proteins encoded by the accessory genes in addition to those encoded by the usual replicative or structural genes, *gag*, *pol* and *env*. These accessory proteins are distinct from those involved in the regulation of gene expression, like those encoded by *tat*, *rev*, *tax* and *rex*. Examples of accessory genes include one or more of *vif*, *vpr*, *vpx*, *vpu* and *nef*. These accessory genes can be found in, for example, HIV (see, for example pages 802 and 803 of "Retroviruses" Ed. Coffin *et al* Pub. CSHL 1997). Non-essential accessory proteins may function in specialised cell types, providing functions that are at least in part duplicative of a function provided by a cellular protein. Typically, the accessory genes are located between *pol* and *env*, just downstream from *env* including the U3 region of the LTR or overlapping portions of the *env* and each other.

The complex retroviruses have evolved regulatory mechanisms that employ virally encoded transcriptional activators as well as cellular transcriptional factors. These *trans*-acting viral proteins serve as activators of RNA transcription directed by the LTRs. The transcriptional *trans*-activators of the lentiviruses are encoded by the viral *tat* genes. Tat binds to a stable, stem-loop, RNA secondary structure, referred to as TAR, one function of which is to apparently optimally position Tat to *trans*-activate transcription.

As mentioned earlier, retroviruses have been proposed as a delivery system (otherwise expressed as a delivery vehicle or delivery vector) for *inter alia* the transfer of a NOI, or a plurality of NOIs, to one or more sites of interest. The transfer can occur *in vitro*, *ex*

vivo, *in vivo*, or combinations thereof. When used in this fashion, the retroviruses are typically called retroviral vectors or recombinant retroviral vectors. Retroviral vectors have even been exploited to study various aspects of the retrovirus life cycle, including receptor usage, reverse transcription and RNA packaging (reviewed by Miller, 1992
5 Curr Top Microbiol Immunol 158:1-24).

In a typical recombinant retroviral vector for use in gene therapy, at least part of one or more of the *gag*, *pol* and *env* protein coding regions may be removed from the virus. This makes the retroviral vector replication-defective. The removed portions may even
10 be replaced by a NOI in order to generate a virus capable of integrating its genome into a host genome but wherein the modified viral genome is unable to propagate itself due to a lack of structural proteins. When integrated in the host genome, expression of the NOI occurs - resulting in, for example, a therapeutic and/or a diagnostic effect. Thus, the transfer of a NOI into a site of interest is typically achieved by: integrating the NOI into
15 the recombinant viral vector; packaging the modified viral vector into a virion coat; and allowing transduction of a site of interest - such as a targeted cell or a targeted cell population.

It is possible to propagate and isolate quantities of retroviral vectors (e.g. to prepare
20 suitable titres of the retroviral vector) for subsequent transduction of, for example, a site of interest by using a combination of a packaging or helper cell line and a recombinant vector.

In some instances, propagation and isolation may entail isolation of the retroviral *gag*,
25 *pol* and *env* genes and their separate introduction into a host cell to produce a "packaging cell line". The packaging cell line produces the proteins required for packaging retroviral DNA but it cannot bring about encapsidation due to the lack of a *psi* region. However, when a recombinant vector carrying a NOI and a *psi* region is introduced into the packaging cell line, the helper proteins can package the *psi*-positive recombinant
30 vector to produce the recombinant virus stock. This can be used to transduce cells to introduce the NOI into the genome of the cells. The recombinant virus whose genome

lacks all genes required to make viral proteins can transduce only once and cannot propagate. These viral vectors which are only capable of a single round of transduction of target cells are known as replication defective vectors. Hence, the NOI is introduced into the host/target cell genome without the generation of potentially harmful retrovirus.

- 5 A summary of the available packaging lines is presented in "Retroviruses" (1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 449).

The design of retroviral packaging cell lines has evolved to address the problem of *inter alia* the spontaneous production of helper virus that was frequently encountered with
10 early designs. As recombination is greatly facilitated by homology, reducing or eliminating homology between the genomes of the vector and the helper has reduced the problem of helper virus production. More recently, packaging cells have been developed in which the *gag*, *pol* and *env* viral coding regions are carried on separate expression plasmids that are independently transfected into a packaging cell line so that
15 three recombinant events are required for wild type viral production. This reduces the potential for production of a replication-competent virus. This strategy is sometimes referred to as the three plasmid transfection method (Soneoka *et al* 1995 Nucl. Acids Res. 23: 628-633).

- 20 Transient transfection can also be used to measure vector production when vectors are being developed. In this regard, transient transfection avoids the longer time required to generate stable vector-producing cell lines and is used if the vector or retroviral packaging components are toxic to cells. Components typically used to generate retroviral vectors include a plasmid encoding the Gag/Pol proteins, a plasmid encoding
25 the Env protein and a plasmid containing a NOI. Vector production involves transient transfection of one or more of these components into cells containing the other required components. If the vector encodes toxic genes or genes that interfere with the replication of the host cell, such as inhibitors of the cell cycle or genes that induce apoptosis, it may be difficult to generate stable vector-producing cell lines, but transient
30 transfection can be used to produce the vector before the cells die. Also, cell lines have been developed using transient infection that produce vector titre levels that are

comparable to the levels obtained from stable vector-producing cell lines (Pear *et al* 1993, Proc Natl Acad Sci 90:8392-8396).

5 In view of the toxicity of some HIV proteins - which can make it difficult to generate stable HIV-based packaging cells - HIV vectors are usually made by transient transfection of vector and helper virus. Some workers have even replaced the HIV Env protein with that of vesicular stomatis virus (VSV). Insertion of the Env protein of VSV facilitates vector concentration as HIV/VSV-G vectors with titres of 5×10^5 (10^8 after concentration) have been generated by transient transfection (Naldini *et al* 1996 Science 10 272: 263-267). Thus, transient transfection of HIV vectors may provide a useful strategy for the generation of high titre vectors (Yee *et al* 1994 PNAS. 91: 9564-9568).

15 With regard to vector titre, the practical uses of retroviral vectors have been limited largely by the titres of transducing particles which can be attained in *in vitro* culture (typically not more than 10^8 particles/ml) and the sensitivity of many enveloped viruses to traditional biochemical and physicochemical techniques for concentrating and purifying viruses.

20 By way of example, several methods for concentration of retroviral vectors have been developed, including the use of centrifugation (Fekete and Cepko 1993 Mol Cell Biol 13: 2604-2613), hollow fibre filtration (Paul *et al* 1993 Hum Gene Ther 4: 609-615) and tangential flow filtration (Kotani *et al* 1994 Hum Gene Ther 5: 19-28). Although a 20-fold increase in viral titre can be achieved, the relative fragility of retroviral Env protein limits the ability to concentrate retroviral vectors and concentrating the virus usually 25 results in a poor recovery of infectious virions. While this problem can be overcome by substitution of the retroviral Env protein with the more stable VSV-G protein, as described above, which allows for more effective vector concentration with better yields, it suffers from the drawback that the VSV-G protein is quite toxic to cells.

30 Although helper-virus free vector titres of 10^7 cfu/ml are obtainable with currently available vectors, experiments can often be done with much lower-titre vector stocks. However, for practical reasons, high-titre virus is desirable, especially when a large

number of cells must be infected. In addition, high titres are a requirement for transduction of a large percentage of certain cell types. For example, the frequency of human hematopoietic progenitor cell infection is strongly dependent on vector titre, and useful frequencies of infection occur only with very high-titre stocks (Hock and Miller 1986 Nature 320: 275-277; Hogge and Humphries 1987 Blood 69: 611-617). In these cases, it is not sufficient simply to expose the cells to a larger volume of virus to compensate for a low virus titre. On the contrary, in some cases, the concentration of infectious vector virions may be critical to promote efficient transduction.

Workers are trying to create high titre vectors for use in gene delivery. By way of example, a comparison of different vector designs has proved useful in helping to define the essential elements required for high-titre viral production. Early work on different retroviral vector design showed that almost all of the internal protein-encoding regions of MLVs could be deleted without abolishing the infectivity of the vector (Miller *et al* 1983 Proc Natl Acad Sci 80: 4709-4713). These early vectors retained only a small portion of the 3' end of the *env*-coding region. Subsequent work has shown that all of the *env*-gene-coding sequences can be removed without further reduction in vector titre (Miller and Rosman 1989 Biotechnology 7: 980-990; Morgenstern and Land 1990 Nucleic Acids Res 18: 3587-3596). Only the viral LTRs and short regions adjoining the LTRs, including the segments needed for plus- and minus-strand DNA priming and a region required for selective packaging of viral RNA into virions (the *psi* site; Mann *et al* 1983 Cell 33: 153-159) were deemed necessary for vector transmission. Nevertheless, viral titres obtained with these early vectors were still about tenfold lower than the parental helper virus titre.

Additional experiments indicated that retention of sequences at the 5' end of the *gag* gene significantly raised viral vector titres and that this was due to an increase in the packaging efficiency of viral RNA into virions (Armentano *et al* 1987 J Virol 61: 1647-1650; Bender *et al* 1987 J Virol 61: 1639-1646; Adam and Miller 1988 J Virol 62: 3802-3806). This effect was not due to viral protein synthesis from the *gag* region of the vector because disruption of the *gag* reading frame or mutating the *gag* codon to a

stop codon had no effect on vector titre (Bender *et al* 1987 *ibid*). These experiments demonstrated that the sequences required for efficient packaging of genomic RNA in MLV were larger than the *psi* signal previously defined by deletion analysis (Mann *et al* 1983 *ibid*). In order to obtain high titres (10^6 to $> 10^7$), it was shown to be important
5 that this larger signal, called *psi* plus, be included in retroviral vectors. It has now been demonstrated that this signal spans from upstream of the splice donor to downstream of the *gag* start codon (Bender *et al* 1987 *ibid*). Because of this position, in spliced *env* expressing transcripts this signal is deleted. This ensures that only full length transcripts containing all three essential genes for viral life cycle are packaged.

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In addition to manipulating the retroviral vector with a view to increasing vector titre, retroviral vectors have also been designed to induce the production of a specific NOI (usually a marker protein) in transduced cells. As already mentioned, the most common retroviral vector design involves the replacement of retroviral sequences with one or
15 more NOIs to create replication-defective vectors. The simplest approach has been to use the promoter in the retroviral 5' LTR to control the expression of a cDNA encoding an NOI or to alter the enhancer/promoter of the LTR to provide tissue-specific expression or inducibility. Alternatively, a single coding region has been expressed by using an internal promoter which permits more flexibility in promoter selection.

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These strategies for expression of a gene of interest have been most easily implemented when the NOI is a selectable marker, as in the case of hypoxanthine-guanine phosphoribosyl transferase (*hprt*) (Miller *et al* 1983 Proc Natl Acad Sci 80: 4709-4713) which facilitates the selection of vector transduced cells. If the vector contains an NOI
25 that is not a selectable marker, the vector can be introduced into packaging cells by co-transfection with a selectable marker present on a separate plasmid. This strategy has an appealing advantage for gene therapy in that a single protein is expressed in the ultimate target cells and possible toxicity or antigenicity of a selectable marker is avoided. However, when the inserted gene is not selectable, this approach has the disadvantage
30 that it is more difficult to generate cells that produce a high titre vector stock. In addition it is usually more difficult to determine the titre of the vector.

The current methodologies used to design retroviral vectors that express two or more proteins have relied on three general strategies. These include: (i) the expression of different proteins from alternatively spliced mRNAs transcribed from one promoter; (ii) the use of the promoter in the 5' LTR and internal promoters to drive transcription of different cDNAs and (iii) the use of internal ribosomal entry site (IRES) elements to allow translation of multiple coding regions from either a single mRNA or from fusion proteins that can then be expressed from an open reading frame.

Vectors containing internal promoters have been widely used to express multiple genes. An internal promoter makes it possible to exploit the promoter/enhancer combinations other than the viral LTR for driving gene expression. Multiple internal promoters can be included in a retroviral vector and it has proved possible to express at least three different cDNAs each from its own promoter (Overell *et al* 1988 Mol Cell Biol 8: 1803-1808).

While there now exist many such modified retroviral vectors which may be used for the expression of NOIs in a variety of mammalian cells, most of these retroviral vectors are derived from simple retroviruses such as murine oncoretroviruses that are incapable of transducing non-dividing cells.

By way of example, a widely used vector that employs alternative splicing to express genes from the viral LTR SV(X) (Cepko *et al* 1984 Cell 37: 1053-1062) contains the neomycin phosphotransferase gene as a selectable marker. The model for this type of vector is the parental virus, MO-MLV, in which the Gag and Gag-Pol proteins are translated from the full-length viral mRNA and the Env protein is made from the spliced mRNA. One of the proteins encoded by the vector is translated from the full-length RNA whereas splicing that links the splice donor near the 5'LTR to a splice acceptor just upstream of the second gene produces an RNA from which the second gene product can be translated. One drawback of this strategy is that foreign sequences are inserted into the intron of the spliced gene. This can affect the ratio of spliced to unspliced RNAs or provide alternative splice acceptors that interfere with production of the spliced RNA

encoding the second gene product (Korman *et al* 1987 Proc Natl Acad Sci 84: 2150-2154). Because these effects are unpredictable, they can affect the production of the encoded genes.

Other modified retroviral vectors can be divided into two classes with regards to splicing capabilities.

The first class of modified retroviral vector, typified by the pBABE vectors (Morgenstern *et al* 1990 Nucleic Acid Research 18: 3587-3596), contain mutations within the splice donor (GT to GC) that inhibit splicing of viral transcripts. Such splicing inhibition is beneficial for two reasons: Firstly, it ensures all viral transcripts contain a packaging signal and thus all can be packaged in the producer cell. Secondly, it prevents potential aberrant splicing between viral splice donors and possible cryptic splice acceptors of inserted genes.

The second class of modified retroviral vector, typified by both N2 (Miller *et al* 1989 Biotechniques 7: 980-990) and the more recent MFG (Dranoff *et al* 1993 Proc Natl Acad Sci 19: 3979-3986), contain functional introns. Both of these vectors use the normal splice donor found within the packaging signal. However, their respective splice acceptors (SAs) differ. For N2, the SA is found within the "extended" packaging signal (Bender *et al* 1987 *ibid*). For MFG, the natural SA (found within *pol*, see Figure 1 thereof) is used. For both these vectors, it has been demonstrated that splicing greatly enhances gene expression in transduced cells (Miller *et al* 1989 *ibid*; Krall *et al* 1996 Gene Therapy 3: 37-48). Such observations support previous findings that, in general, splicing can enhance mRNA translation (Lee *et al* 1981 Nature 294: 228-232; Lewis *et al* 1986 Mol Cell Biol 6: 1998-2010; Chapman *et al* 1991 Nucleic Acids Res 19: 3979-3986). One likely reason for this is that the same machinery involved in transcript splicing may also aid in transcript export from the nucleus.

Unlike the modified retroviral vectors described above, there has been very little work on alternative splicing in the retroviral lentiviral systems which are capable of infecting non-dividing cells (Naldini *et al* 1996 Science 272: 263-267). To date the only

published lentiviral vectors are those derived from HIV-1 (Kim *et al* 1997 J Virol 72: 811-816) and FIV (Poeschla *et al* 1998 Nat Med 4: 354-357). These vectors still contain virally derived splice donor and acceptor sequences (Naldini *et al* 1996 *ibid*).

- 5 Some alternative approaches to developing high titre vectors for gene delivery have included the use of: (i) defective viral vectors such as adenoviruses, adeno-associated virus (AAV), herpes viruses, and pox viruses and (ii) modified retroviral vector designs.

The adenovirus is a double-stranded, linear DNA virus that does not go through an RNA
10 intermediate. There are over 50 different human serotypes of adenovirus divided into 6 subgroups based on the genetic sequence homology. The natural target of adenovirus is the respiratory and gastrointestinal epithelia, generally giving rise to only mild symptoms. Serotypes 2 and 5 (with 95% sequence homology) are most commonly used in adenoviral vector systems and are normally associated with upper respiratory tract infections in the
15 young.

Adenoviruses are nonenveloped, regular icosohedrons. A typical adenovirus comprises a 140nm encapsidated DNA virus. The icosahedral symmetry of the virus is composed of 152 capsomeres: 240 hexons and 12 pentons. The core of the particle contains the 36kb
20 linear duplex DNA which is covalently associated at the 5' ends with the Terminal Protein (TP) which acts as a primer for DNA replication. The DNA has inverted terminal repeats (ITR) and the length of these varies with the serotype.

Entry of adenovirus into cells involves a series of distinct events. Attachment of the virus
25 to the cell occurs via an interaction between the viral fibre (37nm) and the fibre receptors on the cell. This receptor has recently been identified for Ad2/5 serotypes and designated as CAR (Coxsackie and Adeno Receptor, Tomko *et al* (1997 Proc Natl Acad Sci 94: 3352-2258). Internalisation of the virus into the endosome via the cellular $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins is mediated by and viral RGD sequence in the penton-base capsid protein
30 (Wickham *et al.*, 1993 Cell 73: 309-319). Following internalisation, the endosome is disrupted by a process known as endosomolysis, an event which is believed to be

preferentially promoted by the cellular $\alpha\text{v}\beta 5$ integrin (Wickham *et al.*, 1994 J Cell Biol 127: 257-264). In addition, there is recent evidence that the Ad5 fibre knob binds with high affinity to the MHC class 1 $\alpha 2$ domain at the surface of certain cell types including human epithelial and B lymphoblast cells (Hong *et al.*, 1997 EMBO 16: 2294-2306).

5

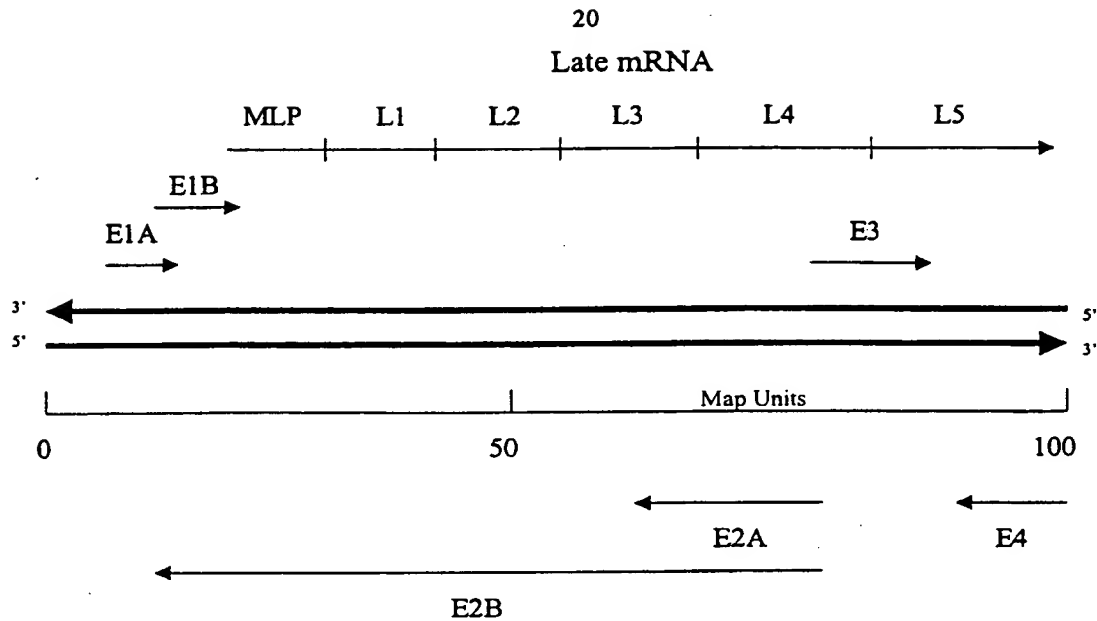
Subsequently the virus is translocated to the nucleus where activation of the early regions occurs and is shortly followed by DNA replication and activation of the late regions. Transcription, replication and packaging of the adenoviral DNA requires both host and viral functional protein machinery.

10

Viral gene expression can be divided into early (E) and late (L) phases. The late phase is defined by the onset of viral DNA replication. Adenovirus structural proteins are generally synthesised during the late phase. Following adenovirus infection, host cellular mRNA and protein synthesis is inhibited in cells infected with most serotypes. The adenovirus lytic cycle with adenovirus 2 and adenovirus 5 is very efficient and results in approximately 10, 000 virions per infected cell along with the synthesis of excess viral protein and DNA that is not incorporated into the virion. Early adenovirus transcription is a complicated sequence of interrelated biochemical events but it entails essentially the synthesis of viral RNAs prior to the onset of DNA replication.

20

The Schematic diagram below is of the adenovirus genome showing the relative direction and position of early and late gene transcription:



The organisation of the adenovirus genome is similar in all of the adenovirus groups and specific functions are generally positioned at identical locations for each serotype studied.

- 5 Early cytoplasmic messenger RNAs are complementary to four defined, noncontiguous regions on the viral DNA. These regions are designated E1-E4. The early transcripts have been classified into an array of intermediate early (E1a), delayed early (E1b, E2a, E2b, E3 and E4), and intermediate regions.
- 10 The early genes are expressed about 6-8 hours after infection and are driven from 7 promoters in gene blocks E1-4.

- The E1a region is involved in transcriptional transactivation of viral and cellular genes as well as transcriptional repression of other sequences. The E1a gene exerts an important control function on all of the other early adenovirus messenger RNAs. In normal tissues, in order to transcribe regions E1b, E2a, E2b, E3 or E4 efficiently, active E1a product is required. However, the E1a function may be bypassed. Cells may be manipulated to provide E1a-like functions or may naturally contain such functions. The virus may also be manipulated to bypass the E1a function. The viral packaging signal overlaps with the E1a enhancer (194-358 nt).
- 15
- 20

The E1b region influences viral and cellular metabolism and host protein shut-off. It also includes the gene encoding the pIX protein (3525-4088 nt) which is required for packaging of the full length viral DNA and is important for the thermostability of the virus. The E1b region is required for the normal progression of viral events late in infection. The E1b product acts in the host nucleus. Mutants generated within the E1b sequences exhibit diminished late viral mRNA accumulation as well as impairment in the inhibition of host cellular transport normally observed late in adenovirus infection. E1b is required for altering functions of the host cell such that processing and transport are shifted in favour of viral late gene products. These products then result in viral packaging and release of virions. E1b produces a 19 kD protein that prevents apoptosis. E1b also produces a 55 kD protein that binds to p53. For a review on adenoviruses and their replication, see WO 96/17053.

The E2 region is essential as it encodes the 72 kDa DNA binding protein, DNA polymerase and the 80 kDa precursor of the 55 kDa Terminal Protein (TP) needed for protein priming to initiate DNA synthesis.

A 19 kDa protein (gp19K) is encoded within the E3 region and has been implicated in modulating the host immune response to the virus. Expression of this protein is upregulated in response to TNF alpha during the first phase of the infection and this then binds and prevents migration of the MHC class I antigens to the epithelial surface, thereby dampening the recognition of the adenoviral infected cells by the cytotoxic T lymphocytes. The E3 region is dispensable in *in vitro* studies and can be removed by deletion of a 1.9 kb *XbaI* fragment.

The E4 region is concerned with decreasing the host protein synthesis and increasing the DNA replication of the virus.

There are 5 families of late genes and all are initiated from the major late promoter. The expression of the late genes includes a very complex post-transcriptional control

mechanism involving RNA splicing. The fibre protein is encoded within the L5 region. The adenoviral genome is flanked by the inverted terminal repeat which in Ad5 is 103 bp and is essential for DNA replication. 30-40 hours post infection viral production is complete.

5

Adenoviruses may be converted for use as vectors for gene transfer by deleting the E1 gene, which is important for the induction of the E2, E3 and E4 promoters. The E1-replication defective virus may be propagated in a cell line that provides the E1 polypeptides in trans, such as the human embryonic kidney cell line 293. A therapeutic gene or genes can be inserted by recombination in place of the E1 gene. Expression of the gene is driven from either the E1 promoter or a heterologous promoter.

10

Even more attenuated adenoviral vectors have been developed by deleting some or all of the E4 open reading frames (ORFs). However, certain second generation vectors appear not to give longer-term gene expression, even though the DNA seems to be maintained. Thus, it appears that the function of one or more of the E4 ORFs may be to enhance gene expression from at least certain viral promoters carried by the virus.

15

An alternative approach to making a more defective virus has been to "gut" the virus completely maintaining only the terminal repeats required for viral replication. The "guttled" or "gutless" viruses can be grown to high titres with a first generation helper virus in the 293 cell line but it has been difficult to separate the "guttled" vector from the helper virus.

20

Replication-competent adenoviruses can also be used for gene therapy. For example, the E1A gene can be inserted into a first generation virus under the regulation of a tumour-specific promoter. In theory, following injection of the virus into a tumour, it could replicated specifically in the tumour but not in the surrounding normal cells. This type of vector could be used either to kill tumour cells directly by lysis or to deliver a "suicide gene" such as the herpes-simplex-virus thymidine-kinase gene (HSV *tk*) which can kill infected and bystander cells following treatment with ganciclovir. Alternatively, an

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adenovirus defective only for E1b has been used specifically for antitumour treatment in phase-1 clinical trials. The polypeptides encoded by E1b are able to block p53-mediated apoptosis, preventing the cell from killing itself in response to viral infection. Thus, in normal nontumour cells, in the absence of E1b, the virus is unable to block apoptosis and is thus unable to produce infectious virus and spread. In tumour cells deficient in p53, the E1b defective virus can grow and spread to adjacent p53-defective tumour cells but not to normal cells. Again, this type of vector could also be used to deliver a therapeutic gene such as HSV *tk*.

- 10 The adenovirus provides advantages as a vector for gene delivery over other gene therapy vector systems for the following reasons:

It is a double stranded DNA nonenveloped virus that is capable of *in vivo* and *in vitro* transduction of a broad range of cell types of human and non-human origin. These cells include respiratory airway epithelial cells, hepatocytes, muscle cells, cardiac myocytes, synoviocytes, primary mammary epithelial cells and post-mitotically terminally differentiated cells such as neurons (with perhaps the important exception of some lymphoid cells including monocytes).

Adenoviral vectors are also capable of transducing non dividing cells. This is very important for diseases, such as cystic fibrosis, in which the affected cells in the lung epithelium, have a slow turnover rate. In fact, several trials are underway utilising adenovirus-mediated transfer of cystic fibrosis transporter (CFTR) into the lungs of afflicted adult cystic fibrosis patients.

Adenoviruses have been used as vectors for gene therapy and for expression of heterologous genes. The large (36 kilobase) genome can accommodate up to 8kb of foreign insert DNA and is able to replicate efficiently in complementing cell lines to produce very high titres of up to 10^{12} . Adenovirus is thus one of the best systems to study the expression of genes in primary non-replicative cells.

30

The expression of viral or foreign genes from the adenovirus genome does not require a

replicating cell. Adenoviral vectors enter cells by receptor mediated endocytosis. Once inside the cell, adenovirus vectors rarely integrate into the host chromosome. Instead, it functions episomally (independently from the host genome) as a linear genome in the host nucleus. Hence the use of recombinant adenovirus alleviates the problems associated with random integration into the host genome.

There is no association of human malignancy with adenovirus infection. Attenuated adenoviral strains have been developed and have been used in humans as live vaccines.

However, current adenoviral vectors suffer from some major limitations for *in vivo* therapeutic use. These include: (i) transient gene expression- the adenoviral vector generally remains episomal and does not replicate so that it is not passed onto subsequent progeny (ii) because of its inability to replicate, target cell proliferation can lead to dilution of the vector (iii) an immunological response raised against the adenoviral proteins so that cells expressing adenoviral proteins, even at a low level, are destroyed and (iv) an inability to achieve an effective therapeutic index since *in vivo* delivery leads to an uptake of the vector and expression of the delivered genes in only a proportion of target cells.

If the features of adenoviruses can be combined with the genetic stability of retro/lentiviruses then essentially the adenovirus can be used to transduce target cells to become transient retroviral producer cells that can stably infect neighbouring cells.

A *SUMMARY OF THE INVENTION*
The present invention seeks to provide a novel retroviral vector.

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In particular, the present invention seeks to provide a novel retroviral vector capable of providing efficient expression of a NOI - or even a plurality of NOIs - at one or more desired target sites.

30 The present invention also seeks to provide a novel system for preparing high titres of vector virion which incorporates safety features for *in vivo* use and which is capable of

providing efficient expression of a NOI - or even a plurality of NOIs - at one or more desired target sites.

According to a first aspect of the present invention, there is provided a retroviral vector comprising a functional splice donor site and a functional splice acceptor site; wherein the functional splice donor site and the functional splice acceptor site flank a first nucleotide sequence of interest ("NOI"); wherein the functional splice donor site is upstream of the functional splice acceptor site; wherein the retroviral vector is derived from a retroviral pro-vector; wherein the retroviral pro-vector comprises a first nucleotide sequence (NS) capable of yielding the functional splice donor site and a second NS capable of yielding the functional splice acceptor site; wherein the first NS is downstream of the second NS; such that the retroviral vector is formed as a result of reverse transcription of the retroviral pro-vector.

According to a second aspect of the present invention, there is provided a retroviral vector wherein the retroviral pro-vector comprises a retroviral packaging signal; and wherein the second NS is located downstream of the retroviral packaging signal such that splicing is preventable at a primary target site.

According to a third aspect of the present invention, there is provided a retroviral vector wherein the second NS is placed downstream of the first NOI such that the first NOI is capable of being expressed at a primary target site.

According to a fourth aspect of the present invention, there is provided a retroviral vector wherein the second NS is placed upstream of a multiple cloning site such that one or more additional NOIs may be inserted.

According to a fifth aspect of the present invention, there is provided a retroviral vector wherein the second NS is a nucleotide sequence coding for an immunological molecule or a part thereof.

According to a sixth aspect of the present invention, there is provided a retroviral vector wherein the immunological molecule is an immunoglobulin.

According to a seventh aspect of the present invention, there is provided a retroviral vector wherein the second NS is a nucleotide sequence coding for an immunoglobulin heavy chain variable region.

According to a eighth aspect of the present invention, there is provided a retroviral vector wherein the vector additionally comprises a functional intron.

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According to a ninth aspect of the present invention, there is provided a retroviral vector wherein the functional intron is positioned so that it is capable of restricting expression of at least one of the NOIs in a desired target site.

According to a tenth aspect of the present invention, there is provided a retroviral vector wherein the target site is a cell.

According to a eleventh aspect of the present invention, there is provided a retroviral vector wherein the vector or pro-vector is derivable from a murine oncoretrovirus or a lentivirus.

20

According to a twelfth aspect of the present invention, there is provided a retroviral vector wherein the vector is derivable from MMLV, MSV, MMTV, HIV-1 or EIAV.

According to a thirteenth aspect of the present invention, there is provided a retroviral vector wherein the retroviral vector is an integrated provirus.

25

According to a fourteenth aspect of the present invention, there is provided a retroviral particle obtainable from a retroviral vector.

30

According to a fifteenth aspect of the present invention, there is provided a cell transfected or transduced with a retroviral vector.

5 According to a sixteenth aspect of the present invention there is provided a retroviral vector or a viral particle or a cell for use in medicine.

According to a seventeenth aspect of the present invention there is provided a retroviral vector or a viral particle or a cell for the manufacture of a pharmaceutical composition to deliver one or more NOIs to a target site in need of same.

10

According to an eighteenth aspect of the present invention there is provided a method comprising transfecting or transducing a cell with a retroviral vector or a viral particle or by use of a cell.

15 According to a nineteenth aspect of the present invention there is provided a delivery system for a retroviral vector or a viral particle or a cell wherein the delivery system comprises one or more non-retroviral expression vector(s), adenoviruse(s), or plasmid(s) or combinations thereof for delivery of an NOI or a plurality of NOIs to a first target cell and a retroviral vector for delivery of an NOI or a plurality of NOIs to a second
20 target cell.

According to a twentieth aspect of the present invention there is provided a retroviral pro-vector.

25 According to a twenty first aspect of the present invention there is provided the use of a functional intron to restrict expression of one or more NOIs within a desired target cell.

According to a twenty second aspect of the present invention there is provided the use of a reverse transcriptase to deliver a first NS from the 3' end of a retroviral pro-vector to
30 the 5' end of a retroviral vector.

According to a twenty third aspect of the present invention there is provided a hybrid viral vector system for *in vivo* gene delivery, which system comprises one or more primary viral vectors which encode a secondary viral vector, the primary vector or vectors capable of infecting a first target cell and of expressing therein the secondary viral vector, which
5 secondary vector is capable of transducing a secondary target cell.

According to a twenty fourth aspect of the present invention there is provided a hybrid viral vector system wherein the primary vector is obtainable from or is based on a adenoviral vector and/or the secondary viral vector is obtainable from or is based on a
10 retroviral vector preferably a lentiviral vector.

According to a twenty fifth aspect of the present invention there is provided a hybrid viral vector system wherein the lentiviral vector comprises or is capable of delivering a split-intron configuration.
15

According to a twenty sixth aspect of the present invention there is provided a lentiviral vector system wherein the lentiviral vector comprises or is capable of delivering a split-intron configuration.

20 According to a twenty seventh aspect of the present invention there is provided an adenoviral vector system wherein the adenoviral vector comprises or is capable of delivering a split-intron configuration.

According to a twenty eighth aspect of the present invention there is provided vectors or
25 plasmids basd on or obtained from any one or more of the entities presented as pE1sp1A, pCI-Neo, pE1RevE, pE1HORSE3.1, pE1PEGASUS4, pCI-Rab, pE1Rab.

According to a twenty ninth aspect of the present invention there is provided a retroviral vector capable of differential expression of NOIs in target cells.
30

Another aspect of the present invention includes a hybrid viral vector system for *in vivo* gene delivery, which system comprises a primary viral vector which encodes a secondary viral vector, the primary vector capable of infecting a first target cell and of expressing therein the secondary viral vector, which secondary vector is capable of transducing a secondary target cell, wherein the primary vector is obtainable from or is based on a
5 adenoviral vector and the secondary viral vector is obtainable from or is based on a retroviral vector preferably a lentiviral vector.

Another aspect of the present invention includes a hybrid viral vector system for *in vivo* gene delivery, which system comprises a primary viral vector which encodes a secondary
10 viral vector, the primary vector capable of infecting a first target cell and of expressing therein the secondary viral vector, which secondary vector is capable of transducing a secondary target cell, wherein the primary vector is obtainable from or is based on a adenoviral vector and the secondary viral vector is obtainable from or is based on a
15 retroviral vector preferably a lentiviral vector; wherein the viral vector system comprises a functional splice donor site and a functional splice acceptor site; wherein the functional splice donor site and the functional splice acceptor site flank a first nucleotide sequence of interest ("NOI"); wherein the functional splice donor site is upstream of the functional splice acceptor site; wherein the retroviral vector is derived from a retroviral pro-vector;
20 wherein the retroviral pro-vector comprises a first nucleotide sequence ("NS") capable of yielding the functional splice donor site and a second NS capable of yielding the functional splice acceptor site; wherein the first NS is downstream of the second NS; such that the retroviral vector is formed as a result of reverse transcription of the retroviral pro-vector.

25 Preferably the retroviral pro-vector comprises a third NS that is upstream of the second nucleotide sequence; wherein the third NS is capable of yielding a non-functional splice donor site.

30 Preferably the retroviral vector further comprises a second NOI; wherein the second NOI is downstream of the functional splice acceptor site.

Preferably the retroviral pro-vector comprises the second NOI; wherein the second NOI is downstream of the second nucleotide sequence.

- 5 Preferably the second NOI, or the expression product thereof, is or comprises a therapeutic agent or a diagnostic agent.

Preferably the first NOI, or the expression product thereof, is or comprises any one or more of an agent conferring selectability (e.g. a marker element), a viral essential
10 element, or a part thereof, or combinations thereof.

Preferably the first NS is at or near to the 3' end of a retroviral pro-vector; preferably wherein the 3' end comprises a U3 region and an R region; and preferably wherein the first NS is located between the U3 region and the R region.

- 15 Preferably the U3 region and/or the first NS of the retroviral pro-vector comprises an NS that is a third NOI; wherein the NOI is any one or more of a transcriptional control element, a coding sequence or a part thereof.

- 20 Preferably the first NS is obtainable from a virus.

Preferably the first NS is an intron or a part thereof.

Preferably the intron is obtainable from the small t-intron of SV40 virus.

- 25 Preferably the vector components are regulated. In one preferred aspect of the invention,
the vector components are regulated by hypoxia.

- 30 In another preferred aspect of the invention, the vector components are regulated by tetracycline on/off system.

Thus, the present invention provides a delivery system which utilises a retroviral vector.

DETAILED DESCRIPTION

The retroviral vector of the delivery system of the present invention comprises a functional splice donor site ("FSDS") and a functional splice acceptor site ("FSAS") which flank a first NOI. The retroviral vector is formed as a result of reverse transcription of a retroviral pro-vector which may comprise a plurality of NOIs.

When the FSDS is positioned upstream of the FSAS, any intervening sequence(s) are capable of being spliced. Typically, splicing removes intervening or "intronic" RNA sequences and the remaining "exonic" sequences are ligated to provide continuous sequences for translation.

The splicing process can be pictorially represented as:

Unspliced Form



Splicing



Spliced Form



In this pictorial representation, Y represents the intervening sequence that is removed as a result of splicing.

The natural splicing configuration for retroviral vectors is shown in Figure 27a. The splicing configuration of known vectors is shown in Figure 27b. The Splicing configuration according to the present invention is shown in Figure 27c.

- 5 In accordance with the present invention, if the FSDS is downstream of the FSAS, then splicing cannot occur.

Likewise, if the FSDS is a non-functional splice donor site (NFSDS) and/or the FSAS is a non-functional acceptor acceptor site (NFAS), then splicing cannot occur.

10

An example of a NFSDS is a mutated FSDS such that the FSDS can no longer be recognised by the splicing mechanism.

- 15 In accordance with the present invention, each NS can be any suitable nucleotide sequence. For example, each sequence can be independently DNA or RNA - which may be synthetically prepared or may be prepared by use of recombinant DNA techniques or may be isolated from natural sources or may be combinations thereof. The sequence may be a sense sequence or an antisense sequence. There may be a plurality of sequences, which may be directly or indirectly joined to each other, or combinations thereof.
- 20

- In accordance with the present invention, each NOI can be any suitable nucleotide sequence. For example, each sequence can be independently DNA or RNA - which may be synthetically prepared or may be prepared by use of recombinant DNA techniques or may be isolated from natural sources or may be combinations thereof. The sequence may be a sense sequence or an antisense sequence. There may be a plurality of sequences, which may be directly or indirectly joined to each other, or combinations thereof.
- 25

- 30 The first NOI may include any one or more of the following selectable markers which have been used successfully in retroviral vectors: the bacterial neomycin and hygromycin

phosphotransferase genes which confer resistance to G418 and hygromycin respectively (Palmer *et al* 1987 Proc Natl Acad Sci 84: 1055-1059; Yang *et al* 1987 Mol Cell Biol 7: 3923-3928); a mutant mouse dihydrofolate reductase gene (*dhfr*) which confers resistance to methotrexate (Miller *et al* 1985 Mol Cell Biol 5: 431-437); the bacterial *gpt* gene which allows cells to grow in medium containing mycophenolic acid, xanthine and aminopterin (Mann *et al* 1983 Cell 33: 153-159); the bacterial *hisD* gene which allows cells to grow in medium without histidine but containing histidinol (Danos and Mulligan 1988 Proc Natl Acad Sci 85: 6460-6464); the multidrug resistance gene (*mdr*) which confers resistance to a variety of drugs (Guild *et al* 1988 Proc Natl Acad Sci 85: 1595-1599; Pastan *et al* 1988 Proc Natl Acad Sci 85: 4486-4490) and the bacterial genes which confer resistance to puromycin or phleomycin (Morgenstern and Land 1990 Nucleic Acid Res 18: 3587-3596).

All of these markers are dominant selectable markers and allow chemical selection of most cells expressing these genes. β -galactosidase can also be considered a dominant marker; cells expressing β -galactosidase can be selected by using the fluorescence-activated cell sorter. In fact, any cell surface protein can provide a selectable marker for cells not already making the protein. Cells expressing the protein can be selected by using the fluorescent antibody to the protein and a cell sorter. Other selectable markers that have been included in vectors include the *hprt* and HSV thymidine kinase which allows cells to grow in medium containing hypoxanthine, aminopterin and thymidine.

The first NOI could contain non-coding sequences, for example the retroviral packaging site or non-sense sequences that render the second NOI non-functional in the provector but when they are removed by the splicing the vector the second NOI is revealed for functional expression.

The first NOI may also encode a viral essential element such as *env* encoding the Env protein which can reduce the complexity of production systems. By way of example, in an adenoviral vector, this allows the retroviral vector genome and the envelope to be configured in a single adenoviral vector under the same promoter control thus providing

a simpler system and leaving more capacity in the adenoviral vector for additional sequences. In one aspect, those additional sequences could be the gag-pol cassette itself. Thus in one adenoviral vector one can produce a retroviral vector particle. Previous studies (Feng et al 1997 Nature Biotechnology 15: 866) have required the use of multiple
5 adenoviral vectors.

If the retroviral component includes an *env* nucleotide sequence, then all or part of that sequence can be optionally replaced with all or part of another *env* nucleotide sequence such as, by way of example, the amphotropic Env protein designated 4070A or the
10 influenza haemagglutinin (HA) or the vesicular stomatitis virus G (VSV-G) protein. Replacement of the *env* gene with a heterologous *env* gene is an example of a technique or strategy called pseudotyping. Pseudotyping is not a new phenomenon and examples may be found in WO-A-98/05759, WO-A-98/05754, WO-A-97/17457, WO-A-96/09400, WO-A-91/00047 and Mebatsion *et al* 1997 Cell 90, 841-847.

15

In one preferred aspect, the retroviral vector of the present invention has been pseudotyped. In this regard, pseudotyping can confer one or more advantages. For example, with the lentiviral vectors, the *env* gene product of the HIV based vectors would restrict these vectors to infecting only cells that express a protein called CD4.
20 But if the *env* gene in these vectors has been substituted with *env* sequences from other RNA viruses, then they may have a broader infectious spectrum (Verma and Somia 1997 Nature 389:239-242). By way of example, workers have pseudotyped an HIV based vector with the glycoprotein from VSV (Verma and Somia 1997 *ibid*).

25 In another alternative, the Env protein may be a modified Env protein such as a mutant or engineered Env protein. Modifications may be made or selected to introduce targeting ability or to reduce toxicity or for another purpose (Valsesia-Wittman *et al* 1996 J Virol 70: 2056-64; Nilson *et al* 1996 Gene Therapy 3: 280-6; Fielding *et al* 1998 Blood 9: 1802 and references cited therein).

30

Suitable second NOI coding sequences include those that are of therapeutic and/or diagnostic application such as, but are not limited to: sequences encoding cytokines, chemokines, hormones, antibodies, engineered immunoglobulin-like molecules, a single chain antibody, fusion proteins, enzymes, immune co-stimulatory molecules, immunomodulatory molecules, anti-sense RNA, a transdominant negative mutant of a target protein, a toxin, a conditional toxin, an antigen, a tumour suppressor protein and growth factors, membrane proteins, vasoactive proteins and peptides, anti-viral proteins and ribozymes, and derivatives thereof (such as with an associated reporter group). When included, such coding sequences may be typically operatively linked to a suitable promoter, which may be a promoter driving expression of a ribozyme(s), or a different promoter or promoters.

The second NOI coding sequence may encode a fusion protein or a segment of a coding sequence

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The retroviral vector of the present invention may be used to deliver a second NOI such as a pro-drug activating enzyme to a tumour site for the treatment of a cancer. In each case, a suitable pro-drug is used in the treatment of the individual (such as a patient) in combination with the appropriate pro-drug activating enzyme. An appropriate pro-drug is administered in conjunction with the vector. Examples of pro-drugs include: etoposide phosphate (with alkaline phosphatase, Senter *et al* 1988 Proc Natl Acad Sci 85: 4842-4846); 5-fluorocytosine (with cytosine deaminase, Mullen *et al* 1994 Cancer Res 54: 1503-1506); Doxorubicin-N-p-hydroxyphenoxyacetamide (with Penicillin-V-Amidase, Kerr *et al* 1990 Cancer Immunol Immunother 31: 202-206); Para-N-bis(2-chloroethyl) aminobenzoyl glutamate (with carboxypeptidase G2); Cephalosporin nitrogen mustard carbamates (with β -lactamase); SR4233 (with P450 Reducase); Ganciclovir (with HSV thymidine kinase, Borrelli *et al* 1988 Proc Natl Acad Sci 85: 7572-7576); mustard pro-drugs with nitroreductase (Friedlos *et al* 1997 J Med Chem 40: 1270-1275) and Cyclophosphamide (with P450 Chen *et al* 1996 Cancer Res 56: 1331-1340).

30

The vector of the present invention may be delivered to a target site by a viral or a non-viral vector.

As it is well known in the art, a vector is a tool that allows or facilitates the transfer of an entity from one environment to another. By way of example, some vectors used in recombinant DNA techniques allow entities, such as a segment of DNA (such as a heterologous DNA segment, such as a heterologous cDNA segment), to be transferred into a target cell. Optionally, once within the target cell, the vector may then serve to maintain the heterologous DNA within the cell or may act as a unit of DNA replication. Examples of vectors used in recombinant DNA techniques include plasmids, chromosomes, artificial chromosomes or viruses.

Non-viral delivery systems include but are not limited to DNA transfection methods. Here, transfection includes a process using a non-viral vector to deliver a gene to a target mammalian cell.

Typical transfection methods include electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection, liposomes, immunoliposomes, lipofectin, cationic agent-mediated, cationic facial amphiphiles (CFAs) (Nature Biotechnology 1996 14; 556), and combinations thereof.

Viral delivery systems include but are not limited to adenovirus vector, an adeno-associated viral (AAV) vector, a herpes viral vector, retroviral vector, lentiviral vector, baculoviral vector. Other examples of vectors include *ex vivo* delivery systems, which include but are not limited to DNA transfection methods such as electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection.

The vector delivery system of the present invention may consist of a primary vector manufactured *in vitro* which encodes the genes necessary to produce a secondary vector *in vivo*.

The primary viral vector or vectors may be a variety of different viral vectors, such as retroviral, adenoviral, herpes virus or pox virus vectors, or in the case of multiple primary viral vectors, they may be a mixture of vectors of different viral origin. In whichever case, the primary viral vectors are preferably defective in that they are incapable of independent replication. Thus, they are capable of entering a target cell and delivering the secondary vector sequences, but not of replicating so as to go on to infect further target cells.

In the case where the hybrid viral vector system comprises more than one primary vector to encode the secondary vector, both or all three primary vectors will be used to transfect or transduce a primary target cell population, usually simultaneously.

Preferably, there is a single primary viral vector which encodes all components of the secondary viral vector.

The preferred single or multiple primary viral vectors are adenoviral vectors.

Adenoviral vectors for use in the invention may be derived from a human adenovirus or an adenovirus which does not normally infect humans. Preferably the vectors are derived from adenovirus type 2 or adenovirus type 5 (Ad2 or Ad5) or a mouse adenovirus or an avian adenovirus such as CELO virus (Cotton *et al* 1993 J Virol 67:3777-3785). The vectors may be replication competent adenoviral vectors but are more preferably defective adenoviral vectors. Adenoviral vectors may be rendered defective by deletion of one or more components necessary for replication of the virus. Typically, each adenoviral vector contains at least a deletion in the E1 region. For production of infectious adenoviral vector particles, this deletion may be complemented by passage of the virus in a human embryo fibroblast cell line such as human 293 cell line, containing an integrated copy of the left portion of Ad5, including the E1 gene. The capacity for insertion of heterologous DNA into such vectors can be up to approximately 7kb. Thus such vectors are useful for construction of a system according

to the invention comprising three separate recombinant vectors each containing one of the essential transcription units for construction of the retroviral secondary vector.

Alternative adenoviral vectors are known in the art which contain further deletions in other adenoviral genes and these vectors are also suitable for use in the invention. Several of these second generation adenoviral vectors show reduced immunogenicity (eg E1 + E2 deletions Gorziglia *et al* 1996 J Virol 70: 4173-4178; E1 + E4 deletions Yeh *et al* 1996 J Virol 70: 559-565). Extended deletions serve to provide additional cloning capacity for the introduction of multiple genes in the vector. For example a 25 kb deletion has been described (Lieber *et al* 1996 J Virol 70: 8944-8960) and a cloning vector deleted of all viral genes has been reported (Fisher *et al* 1996 Virology 217: 11-22) which permit the introduction of more than 35 kb of heterologous DNA. Such vectors may be used to generate an adenoviral primary vector according to the invention encoding two or three transcription units for construction of the retroviral secondary vector.

The secondary viral vector is preferably a retroviral vector. The secondary vector is produced by expression of essential genes for assembly and packaging of a defective viral vector particle, within the primary target cells. It is defective in that it is incapable of independent replication. Thus, once the secondary retroviral vector has transduced a secondary target cell, it is incapable of spreading by replication to any further target cells.

The term "retroviral vector" typically includes a retroviral nucleic acid which is capable of infection, but which is not capable, by itself, of replication. Thus it is replication defective. A retroviral vector typically comprises one or more NOI(s), preferably of non-retroviral origin, for delivery to target cells. A retroviral vector may also comprises a functional splice donor site (FSDS) and a functional splice acceptor site (FSAS) so that when the FSDS is upstream of the FSAS, any intervening sequence(s) are capable of being spliced. A retroviral vector may comprise further non-retroviral sequences, such as non-retroviral control sequences in the U3 region which may influence expression of

an NOI(s) once the retroviral vector is integrated as a provirus into a target cell. The retroviral vector need not contain elements from only a single retrovirus. Thus, in accordance with the present invention, it is possible to have elements derivable from two of more different retroviruses or other sources

5

The term "retroviral pro-vector" typically includes a retroviral vector genome as described above but which comprises a first nucleotide sequence (NS) capable of yielding a functional splice donor site (FSDs) and a second NS capable of yielding a functional splice acceptor site (FSAS) such that the first NS is downstream of the second NS so that
10 splicing associated with the first NS and the second NS cannot occur. Upon reverse transcription of the retroviral pro-vector, a retroviral vector is formed.

The term "retroviral vector particle" refers to the packaged retroviral vector, that is preferably capable of binding to and entering target cells. The components of the
15 particle, as already discussed for the vector, may be modified with respect to the wild type retrovirus. For example, the Env proteins in the proteinaceous coat of the particle may be genetically modified in order to alter their targeting specificity or achieve some other desired function.

20 The retroviral vector of this aspect of the invention may be derivable from a murine oncoretrovirus such as MMLV, MSV or MMTV; or may be derivable from a lentivirus such as HIV-1, EIAV; or may be derivable from another retrovirus.

The retroviral vector of the invention can be modified to render the natural splice donor
25 site of the retrovirus non-functional.

The term "modification" includes the silencing or removal of the natural splice donor. Vectors, such as MLV based vectors, which have the splice donor site removed are known in the art. An example of such a vector is pBABE (Morgenstern *et al* 1990 *ibid*).

30

The secondary vector may be produced from expression of essential genes for retroviral vector production encoded in the DNA of the primary vector. Such genes may include a *gag-pol* gene from a retrovirus, an *env* gene from an enveloped virus and a defective retroviral vector containing one or more therapeutic or diagnostic NOI(s). The defective
5 retroviral vector contains in general terms sequences to enable reverse transcription, at least part of a 5' long terminal repeat (LTR), at least part of a 3'LTR and a packaging signal.

If it is desired to render the secondary vector replication defective, that secondary vector
10 may be encoded by a plurality of transcription units, which may be located in a single or in two or more adenoviral or other primary vectors. Thus, there may be a transcription unit encoding the secondary vector genome, a transcription unit encoding *gag-pol* and a transcription unit encoding *env*. Alternatively, two or more of these may be combined. For example, nucleic acid sequences encoding *gag-pol* and *env*, or *env* and the genome,
15 may be combined in a single transcription unit. Ways of achieving this are known in the art.

Transcription units as described herein are regions of nucleic acid containing coding sequences and the signals for achieving expression of those coding sequences
20 independently of any other coding sequences. Thus, each transcription unit generally comprises at least a promoter, an enhancer and a polyadenylation signal.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Monod theory of gene expression.
25

The term "enhancer" includes a DNA sequence which binds to other protein components of the transcription initiation complex and thus facilitates the initiation of transcription directed by its associated promoter.

30 The promoter and enhancer of the transcription units encoding the secondary vector are preferably strongly active, or capable of being strongly induced, in the primary target

cells under conditions for production of the secondary viral vector. The promoter and/or enhancer may be constitutively efficient, or may be tissue or temporally restricted in their activity. Examples of suitable tissue restricted promoters/enhancers are those which are highly active in tumour cells such as a promoter/enhancer from a MUC1 gene, a CEA gene or a 5T4 antigen gene. Examples of temporally restricted promoters/enhancers are those which are responsive to ischaemia and/or hypoxia, such as hypoxia response elements or the promoter/enhancer of a grp78 or a grp94 gene. One preferred promoter-enhancer combination is a human cytomegalovirus (hCMV) major immediate early (MIE) promoter/enhancer combination.

Other preferred additional components include entities enabling efficient expression of an NOI or a plurality of NOIs.

In one preferred aspect of the present invention, there is hypoxia or ischaemia regulatable expression of the secondary vector components. In this regard, hypoxia is a powerful regulator of gene expression in a wide range of different cell types and acts by the induction of the activity of hypoxia-inducible transcription factors such as hypoxia inducible factor-1 (HIF-1; Wang & Semenza 1993 Proc Natl Acad Sci 90:430), which bind to cognate DNA recognition sites, the hypoxia-responsive elements (HREs) on various gene promoters. Dachs *et al* (1997 Nature Med 5: 515) have used a multimeric form of the HRE from the mouse phosphoglycerate kinase-1 (PGK-1) gene (Firth *et al* 1994 Proc Natl Acad Sci 91:6496-6500) to control expression of both marker and therapeutic genes by human fibrosarcoma cells in response to hypoxia *in vitro* and within solid tumours *in vivo* (Dachs *et al ibid*). Alternatively, the fact that marked glucose deprivation is also present in ischaemic areas of tumours can be used to activate heterologous gene expression specifically in tumours. A truncated 632 base pair sequence of the grp 78 gene promoter, known to be activated specifically by glucose deprivation, has also been shown to be capable of driving high level expression of a reporter gene in murine tumours *in vivo* (Gazit *et al* 1995 Cancer Res 55:1660).

An alternative method of regulating the expression of such components is by using the tetracycline on/off system described by Gossen and Bujard (1992 Proc Natl Acad Sci 89: 5547) as described for the production of retroviral *gal*, *pol* and VSV-G proteins by Yoshida *et al* (1997 Biochem Biophys Res Comm 230: 426). Unusually this regulatory system is also used in the present invention to control the production of the pro-vector genome. This ensures that no vector components are expressed from the adenoviral vector in the absence of tetracycline.

Safety features which may be incorporated into the hybrid viral vector system are described below. One or more such features may be present.

The secondary vector is also advantageous for *in vivo* use in that incorporated into it are one or more features which eliminate the possibility of recombination to produce an infectious virus capable of independent replication. Such features were not included in previous published studies (Feng *et al* 1997 *ibid*). In particular, the construction of a retroviral vector from three components as described below was not described by Feng *et al* (*ibid*).

Firstly, sequence homology between the sequences encoding the components of the secondary vector may be avoided by deletion of regions of homology. Regions of homology allow genetic recombination to occur. In a particular embodiment, three transcription units are used to construct a secondary retroviral vector. The first transcription unit contains a retroviral *gag-pol* gene under the control of a non-retroviral promoter and enhancer. The second transcription unit contains a retroviral *env* gene under the control of a non-retroviral promoter and enhancer. The third transcription unit comprises a defective retroviral genome under the control of a non-retroviral promoter and enhancer. In the native retroviral genome, the packaging signal is located such that part of the *gag* sequence is required for proper functioning. Normally when retroviral vector systems are constructed therefrom, the packaging signal, including part of the *gag* gene, remains in the vector genome. In the present case however, the defective retroviral genome contains a minimal packaging signal which does not contain sequences

homologous to *gag* sequences in the first transcription unit. Also, in retroviruses, for example Moloney Murine Leukaemia virus (MMLV), there is a small region of overlap between the 3' end of the *pol* coding sequence and the 5' end of *env*. The corresponding region of homology between the first and second transcription units may be removed by
5 altering the sequence of either the 3' end of the *pol* coding sequence or the 5' end of *env* so as to change the codon usage but not the amino acid sequence of the encoded proteins.

Secondly, the possibility of replication competent secondary viral vectors may be avoided by pseudotyping the genome of one retrovirus with the Env protein of another
10 retrovirus or another enveloped virus so that regions of homology between the *env* and *gag-pol* components are avoided.

In a particular embodiment the retroviral vector is constructed from the following three components: The first transcription unit contains a retroviral *gag-pol* gene under the
15 control of a non-retroviral promoter and enhancer. The second transcription unit contains the *env* gene from the alternative enveloped virus, under the control of a non-retroviral promoter and enhancer. The third transcription unit comprises a defective retroviral genome under the control of a non-retroviral promoter and enhancer. The defective retroviral genome contains a minimal packaging signal which does not contain
20 sequences homologous to *gag* sequences in the first transcription unit.

Thirdly, the possibility of replication competent retroviruses can be eliminated by using two transcription units constructed in a particular way. The first transcription unit contains a *gag-pol* coding region under the control of a promoter-enhancer active in the
25 primary target cell such as a hCMV promoter-enhancer or a tissue restricted promoter-enhancer. The second transcription unit encodes a retroviral genome RNA capable of being packaged into a retroviral particle. The second transcription unit contains retroviral sequences necessary for packaging, integration and reverse transcription and also contains sequences coding for an *env* protein of an enveloped virus and the coding
30 sequence of one or more therapeutic genes.

In this example, the transcription of the *env* and an NOI coding sequences is devised such that the Env protein is preferentially produced in the primary target cell while the NOI expression product is or are preferentially produced in the secondary target cell.

- 5 A suitable intron splicing arrangement is described later on in Example 5 and illustrated in Figure 17 and Figure 27c. Here, a splice donor site is positioned downstream of a splice acceptor site in the retroviral genome sequence delivered by the primary vector to the primary target cell. Splicing will therefore be absent or infrequent in the primary target cell so the Env protein will preferentially be expressed. However, once the vector
- 10 genome has gone through the process of reverse transcription and integration into the secondary target cell, a functional splice donor sequence will be located in the 5' LTR, upstream of a functional splice acceptor sequence. Splicing occurs to splice out the *env* sequence and transcripts of the NOI are produced.
- 15 In a second arrangement of this example, the expression of an NOI is restricted to the secondary target cell and prevented from being expressed in the primary target cell as follows: This arrangement is described later on in Example 6 and illustrated in Figure 18. There, a promoter-enhancer and a first fragment of an NOI containing the 5' end of the coding sequence and a natural or artificially derived or derivable splice donor
- 20 sequence are inserted at the 3' end of the retroviral genome construct upstream of the R-region. A second fragment of the NOI which contains all the sequences required to complete the coding region is placed downstream of a natural or artificially derived or derivable splice acceptor sequence located downstream from the packaging signal in the retroviral genome construct. On reverse transcription and integration of the retroviral
- 25 genome in the secondary target cell, the promoter 5' fragment of the NOI and the functional splice donor sequence are located upstream of the functional splice acceptor and the 3' end of the NOI. Transcription from the promoter and splicing then permit translation of the NOI in the secondary target cell.

In a preferred embodiment the hybrid viral vector system according to the invention comprises single or multiple adenoviral primary vectors which encodes or encode a retroviral secondary vector.

- 5 Preferred embodiments of the present invention described address one of the major problems associated with adenoviral and other viral vectors, namely that gene expression from such vectors is transient. The retroviral particles generated from the primary target cells can transduce secondary target cells and gene expression in the secondary target cells is stably maintained because of the integration of the retroviral vector genome into
- 10 the host cell genome. The secondary target cells do not express significant amounts of viral protein antigens and so are less immunogenic than cells transduced with adenoviral vector.

- The use of a retroviral vector as the secondary vector is advantageous because it allows a
- 15 degree of cellular discrimination, for instance by permitting the targeting of rapidly dividing cells. Furthermore, retroviral integration permits the stable expression of therapeutic genes in the target tissue, including stable expression in proliferating target cells.

- 20 The use of the novel retroviral vector design of the present invention is also advantageous in that gene expression can be limited to a primary or a secondary target site. In this way, single or multiple NOIs can be preferentially expressed at a secondary target site and poorly expressed or not expressed at a biologically significant level at a primary target site. As a result, the possible toxicity or antigenicity of an NOI may be
- 25 avoided.

Preferably, the primary viral vector preferentially transduces a certain cell type or cell types.

More preferably, the primary vector is a targeted vector, that is it has a tissue tropism which is altered compared to the native virus, so that the vector is targeted to particular cells.

- 5 The term "targeted vector" is not necessarily linked to the term "target site" or target cell".

"Target site" refers to a site which a vector, whether native or targeted, is capable of transfecting or transducing.

10

"Primary target site" refers to a first site which a vector, whether native or targeted, is capable of transfecting or transducing.

15

"Secondary target site" refers to a second site which a vector, whether native or targeted, is capable of transfecting or transducing.

"Target cell" simply refers to a cell which a vector, whether native or targeted, is capable of transfecting or transducing.

20

"Primary target cell" refers to a first cell which a vector, whether native or targeted, is capable of transfecting or transducing.

"Secondary target cell" refers to a second cell which a vector, whether native or targeted, is capable of transfecting or transducing.

25

The preferred, adenoviral primary vector according to the invention is also preferably a targeted vector, in which the tissue tropism of the vector is altered from that of a wild-type adenovirus. Adenoviral vectors can be modified to produce targeted adenoviral vectors for example as described in: Krasnykh *et al* 1996 J. Virol 70: 6839-6846;
30 Wickham *et al* 1996 J. Virol 70: 6831-6838; Stevenson *et al* 1997 J. Virol 71: 4782-

4790; Wickham *et al* 1995 Gene Therapy 2: 750-756; Douglas *et al* 1997 Neuromuscul. Disord 7:284-298; Wickham *et al* 1996 Nature Biotechnology 14: 1570-1573.

Primary target cells for the vector system according to the invention include
5 haematopoietic cells (including monocytes, macrophages, lymphocytes, granulocytes or progenitor cells of any of these); endothelial cells; tumour cells; stromal cells; astrocytes or glial cells; muscle cells; and epithelial cells.

Thus, a primary target cell according to the invention, capable of producing the second
10 viral vector, may be of any of the above cell types.

In a preferred embodiment, the primary target cell according to the invention is a monocyte or macrophage transduced by a defective adenoviral vector containing a first transcription unit for a retroviral *gag-pol* and a second transcription unit capable of
15 producing a packageable defective retroviral genome. In this case at least the second transcription unit is preferably under the control of a promoter-enhancer which is preferentially active in a diseased location within the body such as an ischaemic site or the micro-environment of a solid tumour.

20 In a particularly preferred embodiment, the second transcription unit is constructed such that on insertion of the genome into the secondary target cell, an intron is generated which serves to reduce expression of a viral essential element, such as the viral *env* gene, and permit efficient expression of a therapeutic and/or diagnostic NOI or NOIs.

25 The packaging cell may be an *in vivo* packaging cell in the body of an individual to be treated or it may be a cell cultured *in vitro* such as a tissue culture cell line. Suitable cell lines include mammalian cells such as murine fibroblast derived cell lines or human cell lines. Preferably the packaging cell line is a human cell line, such as for example: HEK293, 293-T, TE671, HT1080.

Alternatively, the packaging cell may be a cell derived from the individual to be treated such as a monocyte, macrophage, blood cell or fibroblast. The cell may be isolated from an individual and the packaging and vector components administered *ex vivo* followed by re-administration of the autologous packaging cells. Alternatively the packaging and vector components may be administered to the packaging cell *in vivo*. Methods for introducing retroviral packaging and vector components into cells of an individual are known in the art. For example, one approach is to introduce the different DNA sequences that are required to produce a retroviral vector particle e.g. the *env* coding sequence, the *gag-pol* coding sequence and the defective retroviral genome into the cell simultaneously by transient triple transfection (Landau & Littman 1992 J. Virol. 66, 5110; Soneoka *et al* 1995 Nucleic Acids Res 23:628-633).

The secondary viral vectors may also be targeted vectors. For retroviral vectors, this may be achieved by modifying the Env protein. The Env protein of the retroviral secondary vector needs to be a non-toxic envelope or an envelope which may be produced in non-toxic amounts within the primary target cell, such as for example a MMLV amphotropic envelope or a modified amphotropic envelope. The safety feature in such a case is preferably the deletion of regions or sequence homology between retroviral components.

Preferably the envelope is one which allows transduction of human cells. Examples of suitable *env* genes include, but are not limited to, VSV-G, a MLV amphotropic *env* such as the 4070A *env*, the RD114 feline leukaemia virus *env* or haemagglutinin (HA) from an influenza virus. The Env protein may be one which is capable of binding to a receptor on a limited number of human cell types and may be an engineered envelope containing targeting moieties. The *env* and *gag-pol* coding sequences are transcribed from a promoter and optionally an enhancer active in the chosen packaging cell line and the transcription unit is terminated by a polyadenylation signal. For example, if the packaging cell is a human cell, a suitable promoter-enhancer combination is that from the human cytomegalovirus major immediate early (hCMV-MIE) gene and a polyadenylation

signal from SV40 virus may be used. Other suitable promoters and polyadenylation signals are known in the art.

5 The secondary target cell population may be the same as the primary target cell population. For example delivery of a primary vector of the invention to tumour cells leads to replication and generation of further vector particles which can transduce further tumour cells.

10 Alternatively, the secondary target cell population may be different from the primary target cell population. In this case the primary target cells serve as an endogenous factory within the body of the treated individual and produce additional vector particles which can transduce the secondary target cell population. For example, the primary target cell population may be haematopoietic cells transduced by the primary vector *in vivo* or *ex vivo*. The primary target cells are then delivered to or migrate to a site within
15 the body such as a tumour and produce the secondary vector particles, which are capable of transducing for example mitotically active tumour cells within a solid tumour.

The retroviral vector particle according to the invention will also be capable of transducing cells which are slowly-dividing, and which non-lentiviruses such as MLV
20 would not be able to efficiently transduce. Slowly-dividing cells divide once in about every three to four days including certain tumour cells. Although tumours contain rapidly dividing cells, some tumour cells especially those in the centre of the tumour, divide infrequently. Alternatively the target cell may be a growth-arrested cell capable of undergoing cell division such as a cell in a central portion of a tumour mass or a stem
25 cell such as a haematopoietic stem cell or a CD34-positive cell. As a further alternative, the target cell may be a precursor of a differentiated cell such as a monocyte precursor, a CD33-positive cell, or a myeloid precursor. As a further alternative, the target cell may be a differentiated cell such as a neuron, astrocyte, glial cell, microglial cell, macrophage, monocyte, epithelial cell, endothelial cell, hepatocyte, spermatocyte,
30 spermatid or spermatozoa. Target cells may be transduced either *in vitro* after isolation from a human individual or may be transduced directly *in vivo*.

The invention permits the localised production of high titres of defective retroviral vector particles *in vivo* at or near the site at which action of a therapeutic protein or proteins is required with consequent efficient transduction of secondary target cells. This is more
5 efficient than using either a defective adenoviral vector or a defective retroviral vector alone.

The invention also permits the production of retroviral vectors such as MMLV-based vectors in non-dividing and slowly-dividing cells *in vivo*. It had previously been possible
10 to produce MMLV-based retroviral vectors only in rapidly dividing cells such as tissue culture-adapted cells proliferating *in vitro* or rapidly dividing tumour cells *in vivo*. Extending the range of cell types capable of producing retroviral vectors is advantageous for delivery of genes to the cells of solid tumours, many of which are dividing slowly, and for the use of non-dividing cells such as endothelial cells and cells of various
15 haematopoietic lineages as endogenous factories for the production of therapeutic protein products.

The delivery of one or more therapeutic genes by a vector system according to the present invention may be used alone or in combination with other treatments or
20 components of the treatment.

For example, the retroviral vector of the present invention may be used to deliver one or more NOI(s) useful in the treatment of the disorders listed in WO-A-98/05635. For ease of reference, part of that list is now provided: cancer, inflammation or inflammatory
25 disease, dermatological disorders, fever, cardiovascular effects, haemorrhage, coagulation and acute phase response, cachexia, anorexia, acute infection, HIV infection, shock states, graft-versus-host reactions, autoimmune disease, reperfusion injury, meningitis, migraine and aspirin-dependent anti-thrombosis; tumour growth, invasion and spread, angiogenesis, metastases, malignant, ascites and malignant pleural effusion;
30 cerebral ischaemia, ischaemic heart disease, osteoarthritis, rheumatoid arthritis, osteoporosis, asthma, multiple sclerosis, neurodegeneration, Alzheimer's disease,

atherosclerosis, stroke, vasculitis, Crohn's disease and ulcerative colitis; periodontitis, gingivitis; psoriasis, atopic dermatitis, chronic ulcers, epidermolysis bullosa; corneal ulceration, retinopathy and surgical wound healing; rhinitis, allergic conjunctivitis, eczema, anaphylaxis; restenosis, congestive heart failure, endometriosis, atherosclerosis
5 or endosclerosis.

In addition, or in the alternative, the retroviral vector of the present invention may be used to deliver one or more NOI(s) useful in the treatment of disorders listed in WO-A-98/07859. For ease of reference, part of that list is now provided: cytokine and cell
10 proliferation/differentiation activity; immunosuppressant or immunostimulant activity (e.g. for treating immune deficiency, including infection with human immune deficiency virus; regulation of lymphocyte growth; treating cancer and many autoimmune diseases, and to prevent transplant rejection or induce tumour immunity); regulation of haematopoiesis, e.g. treatment of myeloid or lymphoid diseases; promoting growth of
15 bone, cartilage, tendon, ligament and nerve tissue, e.g. for healing wounds, treatment of burns, ulcers and periodontal disease and neurodegeneration; inhibition or activation of follicle-stimulating hormone (modulation of fertility); chemotactic/chemokinetic activity (e.g. for mobilising specific cell types to sites of injury or infection); haemostatic and thrombolytic activity (e.g. for treating haemophilia and stroke); antiinflammatory activity
20 (for treating e.g. septic shock or Crohn's disease); as antimicrobials; modulators of e.g. metabolism or behaviour; as analgesics; treating specific deficiency disorders; in treatment of e.g. psoriasis, in human or veterinary medicine.

In addition, or in the alternative, the retroviral vector of the present invention may be
25 used to deliver one or more NOI(s) useful in the treatment of disorders listed in WO-A-98/09985. For ease of reference, part of that list is now provided: macrophage inhibitory and/or T cell inhibitory activity and thus, anti-inflammatory activity; anti-immune activity, i.e. inhibitory effects against a cellular and/or humoral immune response, including a response not associated with inflammation; inhibit the ability of
30 macrophages and T cells to adhere to extracellular matrix components and fibronectin, as well as up-regulated fas receptor expression in T cells; inhibit unwanted immune reaction

and inflammation including arthritis, including rheumatoid arthritis, inflammation associated with hypersensitivity, allergic reactions, asthma, systemic lupus erythematosus, collagen diseases and other autoimmune diseases, inflammation associated with atherosclerosis, arteriosclerosis, atherosclerotic heart disease, reperfusion injury, cardiac arrest, myocardial infarction, vascular inflammatory disorders, respiratory distress syndrome or other cardiopulmonary diseases, inflammation associated with peptic ulcer, ulcerative colitis and other diseases of the gastrointestinal tract, hepatic fibrosis, liver cirrhosis or other hepatic diseases, thyroiditis or other glandular diseases, glomerulonephritis or other renal and urologic diseases, otitis or other oto-rhino-laryngological diseases, dermatitis or other dermal diseases, periodontal diseases or other dental diseases, orchitis or epididymo-orchitis, infertility, orchidial trauma or other immune-related testicular diseases, placental dysfunction, placental insufficiency, habitual abortion, eclampsia, pre-eclampsia and other immune and/or inflammatory-related gynaecological diseases, posterior uveitis, intermediate uveitis, anterior uveitis, conjunctivitis, chorioretinitis, uveoretinitis, optic neuritis, intraocular inflammation, e.g. retinitis or cystoid macular oedema, sympathetic ophthalmia, scleritis, retinitis pigmentosa, immune and inflammatory components of degenerative fondus disease, inflammatory components of ocular trauma, ocular inflammation caused by infection, proliferative vitreo-retinopathies, acute ischaemic optic neuropathy, excessive scarring, e.g. following glaucoma filtration operation, immune and/or inflammation reaction against ocular implants and other immune and inflammatory-related ophthalmic diseases, inflammation associated with autoimmune diseases or conditions or disorders where, both in the central nervous system (CNS) or in any other organ, immune and/or inflammation suppression would be beneficial, Parkinson's disease, complication and/or side effects from treatment of Parkinson's disease, AIDS-related dementia complex HIV-related encephalopathy, Devic's disease, Sydenham chorea, Alzheimer's disease and other degenerative diseases, conditions or disorders of the CNS, inflammatory components of stokes, post-polio syndrome, immune and inflammatory components of psychiatric disorders, myelitis, encephalitis, subacute sclerosing pan-encephalitis, encephalomyelitis, acute neuropathy, subacute neuropathy, chronic neuropathy, Guillain-Barre syndrome, Sydenham chora, myasthenia gravis,

pseudo-tumour cerebri, Down's Syndrome, Huntington's disease, amyotrophic lateral sclerosis, inflammatory components of CNS compression or CNS trauma or infections of the CNS, inflammatory components of muscular atrophies and dystrophies, and immune and inflammatory related diseases, conditions or disorders of the central and peripheral nervous systems, post-traumatic inflammation, septic shock, infectious diseases, inflammatory complications or side effects of surgery, bone marrow transplantation or other transplantation complications and/or side effects, inflammatory and/or immune complications and side effects of gene therapy, e.g. due to infection with a viral carrier, or inflammation associated with AIDS, to suppress or inhibit a humoral and/or cellular immune response, to treat or ameliorate monocyte or leukocyte proliferative diseases, e.g. leukaemia, by reducing the amount of monocytes or lymphocytes, for the prevention and/or treatment of graft rejection in cases of transplantation of natural or artificial cells, tissue and organs such as cornea, bone marrow, organs, lenses, pacemakers, natural or artificial skin tissue.

Further provided according to the invention are methods of controlling production of a therapeutic NOI or NOIs such that the therapeutic NOI or NOIs is/are preferentially expressed in a secondary target cell population and is/are poorly expressed or not expressed at a biologically significant level in a primary target cell.

The present invention also provides a pharmaceutical composition for treating an individual by gene therapy, wherein the composition comprises a therapeutically effective amount of the retroviral vector of the present invention comprising one or more deliverable therapeutic and/or diagnostic NOI(s) or a viral particle produced by or obtained from same. The pharmaceutical composition may be for human or animal usage. Typically, a physician will determine the actual dosage which will be most suitable for an individual subject and it will vary with the age, weight and response of the particular individual.

The composition may optionally comprise a pharmaceutically acceptable carrier, diluent, excipient or adjuvant. The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical

practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s), and other carrier agents that may aid or increase the viral entry into the target site (such as for example a lipid delivery system).

5

Where appropriate, the pharmaceutical compositions can be administered by any one or more of: inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules
10 either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example
15 enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

In a further aspect of the present invention, there is provided a hybrid viral vector system
20 in the general sense (i.e. not necessarily limited to the aforementioned first aspect of the present invention as defined above) for *in vivo* gene delivery, which system comprises one or more primary viral vectors which encode a secondary viral vector, the primary vector or vectors capable of infecting a first target cell and of expressing therein the secondary viral vector, which secondary vector is capable of transducing a secondary target cell.

25

With this particular embodiment, the genetic vector of the invention is thus a hybrid viral vector system for gene delivery which is capable of generation of defective infectious particles from within a target cell. Thus a genetic vector of the invention consists of a primary vector manufactured *in vitro* which encodes the genes necessary to produce a
30 secondary vector *in vivo*. In use, the secondary vector carries one or more selected genes

for insertion into the secondary target cell. The selected genes may be one or more marker genes and/or therapeutic genes. Marker genes encode selectable and/or detectable proteins.

More aspects concerning this particular aspect of the present invention now follow - which
5 teachings are also applicable to the aforementioned aspects of the present invention.

In another aspect the invention provides target cells infected by the primary viral vector or vectors and capable of producing infectious secondary viral vector particles.

10 In a further aspect the invention provides a method of treatment of a human or non-human mammal, which method comprises administering a hybrid viral vector system or target cells infected by the primary viral vector or vectors, as described herein.

The primary viral vector or vectors may be a variety of different viral vectors, such as
15 retroviral, adenoviral, herpes virus or pox virus vectors, or in the case of multiple primary viral vectors, they may be a mixture of vectors of different viral origin. In whichever case, the primary viral vectors are preferably defective in that they are incapable of independent replication. Thus, they are capable of entering a target cell and delivering the secondary vector sequences, but not of replicating so as to go on to infect further target cells.

20 In the case where the hybrid viral vector system comprises more than one primary vector to encode the secondary vector, both or all three primary vectors will be used to infect a primary target cell population, usually simultaneously. Preferably, there is a single primary viral vector which encodes all components of the secondary viral vector.

25 The preferred single or multiple primary viral vectors are adenoviral vectors. Adenovirus vectors have significant advantages over other viral vectors in terms of the titres which can be obtained from *in vitro* cultures. The adenoviral particles are also comparatively stable compared with those of enveloped viruses and are therefore more readily purified and
30 stored. However, current adenoviral vectors suffer from major limitations for *in vivo* therapeutic use since gene expression from defective adenoviral vectors is only transient.

Because the vector genome does not replicate, target cell proliferation leads to dilution of the vector. Also cells expressing adenoviral proteins, even at a low level, are destroyed by an immunological response raised against the adenoviral proteins.

- 5 The secondary viral vector is preferably a retroviral vector. The secondary vector is produced by expression of essential genes for assembly and packaging of a defective viral vector particle, within the primary target cells. It is defective in that it is incapable of independent replication. Thus, once the secondary retroviral vector has transduced a secondary target cell, it is incapable of spreading by replication to any further target cells.

10

- The secondary vector may be produced from expression of essential genes for retroviral vector production encoded in the DNA of the primary vector. Such genes may include a gag-pol gene from a retrovirus, an envelope gene from an enveloped virus and a defective retroviral genome containing one or more therapeutic genes. The defective retroviral genome contains in general terms sequences to enable reverse transcription, at least part of a 5' long terminal repeat (LTR), at least part of a 3'LTR and a packaging signal.

15

- Importantly, the secondary vector is also safe for *in vivo* use in that incorporated into it are one or more safety features which eliminate the possibility of recombination to produce an infectious virus capable of independent replication.

20

- To ensure that it is replication defective the secondary vector may be encoded by a plurality of transcription units, which may be located in a single or in two or more adenoviral or other primary vectors. Thus, there may be a transcription unit encoding the secondary vector genome, a transcription unit encoding *gag-pol* and a transcription unit encoding *env*. Alternatively, two or more of these may be combined. For example, nucleic acid sequences encoding *gag-pol* and *env*, or *env* and the genome, may be combined in a single transcription unit. Ways of achieving this are known in the art.

25

- 30 Transcription units as described herein are regions of nucleic acid containing coding sequences and the signals for achieving expression of those coding sequences

independently of any other coding sequences. Thus, each transcription unit generally comprises at least a promoter, an enhancer and a polyadenylation signal. The promoter and enhancer of the transcription units encoding the secondary vector are preferably strongly active, or capable of being strongly induced, in the primary target cells under conditions for production of the secondary viral vector. The promoter and/or enhancer may be constitutively efficient, or may be tissue or temporally restricted in their activity. Examples of suitable tissue restricted promoters/enhancers are those which are highly active in tumour cells such as a promoter/enhancer from a MUC1 gene, a CEA gene or a 5T4 antigen gene. Examples of temporally restricted promoters/enhancers are those which are responsive to ischaemia and/or hypoxia, such as hypoxia response elements or the promoter/enhancer of a *grp78* or a *grp94* gene. One preferred promoter-enhancer combination is a human cytomegalovirus (hCMV) major immediate early (MIE) promoter/enhancer combination.

Hypoxia or ischaemia regulatable expression of secondary vector components may be particularly useful under certain circumstances. Hypoxia is a powerful regulator of gene expression in a wide range of different cell types and acts by the induction of the activity of hypoxia-inducible transcription factors such as hypoxia inducible factor-1 (HIF-1; Wang & Semenza (1993). *Proc. Natl. Acad. Sci USA* 90:430), which bind to cognate DNA recognition sites, the hypoxia-responsive elements (HREs) on various gene promoters. Dachs *et al* (1997). *Nature Med.* 5: 515.) have used a multimeric form of the HRE from the mouse phosphoglycerate kinase-1 (PGK-1) gene (Firth *et al.* (1994). *Proc. Natl. Acad. Sci USA* 91:6496-6500) to control expression of both marker and therapeutic genes by human fibrosarcoma cells in response to hypoxia *in vitro* and within solid tumours *in vivo* (Dachs *et al* *ibid*). Alternatively, the fact that marked glucose deprivation is also present in ischaemic areas of tumours can be used to activate heterologous gene expression specifically in tumours. A truncated 632 base pair sequence of the *grp 78* gene promoter, known to be activated specifically by glucose deprivation, has also been shown to be capable of driving high level expression of a reporter gene in murine tumours *in vivo* (Gazit G, *et al* (1995). *Cancer Res.* 55:1660).

Safety features which may be incorporated into the hybrid viral vector system are described below. One or more such features may be present.

Firstly, sequence homology between the sequences encoding the components of the secondary vector may be avoided by deletion of regions of homology. Regions of homology allow genetic recombination to occur. In a particular embodiment, three transcription units are used to construct a secondary retroviral vector. A first transcription unit contains a retroviral *gag-pol* gene under the control of a non-retroviral promoter and enhancer. A second transcription unit contains a retroviral *env* gene under the control of a non-retroviral promoter and enhancer. A third transcription unit comprises a defective retroviral genome under the control of a non-retroviral promoter and enhancer. In the native retroviral genome, the packaging signal is located such that part of the *gag* sequence is required for proper functioning. Normally when retroviral vector systems are constructed therefore, the packaging signal, including part of the *gag* gene, remains in the vector genome. In the present case however, the defective retroviral genome contains a minimal packaging signal which does not contain sequences homologous to *gag* sequences in the first transcription unit. Also, in retroviruses, for example Moloney Murine Leukaemia virus (MMLV), there is a small region of overlap between the 3' end of the *pol* coding sequence and the 5' end of *env*. The corresponding region of homology between the first and second transcription units may be removed by altering the sequence of either the 3' end of the *pol* coding sequence or the 5' end of *env* so as to change the codon usage but not the amino acid sequence of the encoded proteins.

Secondly, the possibility of replication competent secondary viral vectors may be avoided by pseudotyping the genome of one retrovirus with the envelope protein of another retrovirus or another enveloped virus so that regions of homology between the *env* and *gag-pol* components are avoided. In a particular embodiment the retroviral vector is constructed from the following three components. The first transcription unit contains a retroviral *gag-pol* gene under the control of a non-retroviral promoter and enhancer. The second transcription unit contains the *env* gene from the alternative enveloped virus, under the control of a non-retroviral promoter and enhancer. The third transcription unit

comprises a defective retroviral genome under the control of a non-retroviral promoter and enhancer. The defective retroviral genome contains a minimal packaging signal which does not contain sequences homologous to *gag* sequences in the first transcription unit.

- 5 Pseudotyping may involve for example a retroviral genome based on a lentivirus such as an HIV or equine infectious anaemia virus (EIAV) and the envelope protein may for example be the amphotropic envelope protein designated 4070A. Alternatively, the retroviral genome may be based on MMLV and the envelope protein may be a protein from another virus which can be produced in non-toxic amounts within the primary target cell such as an
- 10 Influenza haemagglutinin or vesicular stomatitis virus G protein. In another alternative, the envelope protein may be a modified envelope protein such as a mutant or engineered envelope protein. Modifications may be made or selected to introduce targeting ability or to reduce toxicity or for another purpose.
- 15 Thirdly, the possibility of replication competent retroviruses can be eliminated by using two transcription units constructed in a particular way. The first transcription unit contains a *gag-pol* coding region under the control of a promoter-enhancer active in the primary target cell such as a hCMV promoter-enhancer or a tissue restricted promoter-enhancer. The second transcription unit encodes a retroviral genome RNA capable of being packaged
- 20 into a retroviral particle. The second transcription unit contains retroviral sequences necessary for packaging, integration and reverse transcription and also contains sequences coding for an *env* protein of an enveloped virus and the coding sequence of one or more therapeutic genes.
- 25 In a preferred embodiment the hybrid viral vector system according to the invention comprises single or multiple adenoviral primary vectors which encodes or encode a retroviral secondary vector. Adenoviral vectors for use in the invention may be derived from a human adenovirus or an adenovirus which does not normally infect humans. Preferably the vectors are derived from Adenovirus Type 2 or adenovirus Type 5 (Ad2 or
- 30 Ad5) or a mouse adenovirus or an avian adenovirus such as CELO virus (Cotton et al 1993 J. Virol. 67:3777-3785). The vectors may be replication competent adenoviral vectors but

are more preferably defective adenoviral vectors. Adenoviral vectors may be rendered defective by deletion of one or more components necessary for replication of the virus. Typically, each adenoviral vector contains at least a deletion in the E1 region. For production of infectious adenoviral vector particles, this deletion may be complemented by
5 passage of the virus in a human embryo fibroblast cell line such as human 293 cell line, containing an integrated copy of the left portion of Ad5, including the E1 gene. The capacity for insertion of heterologous DNA into such vectors can be up to approximately 7kb. Thus such vectors are useful for construction of a system according to the invention comprising three separate recombinant vectors each containing one of the essential
10 transcription units for construction of the retroviral secondary vector.

Alternative adenoviral vectors are known in the art which contain further deletions in other adenoviral genes and these vectors are also suitable for use in the invention. Several of these second generation adenoviral vectors show reduced immunogenicity (eg E1 + E2
15 deletions Gorziglia et al 1996 J. Virol. 70: 4173-4178; E1 + E4 deletions Yeh et al 1996 J. Virol. 70: 559-565). Extended deletions serve to provide additional cloning capacity for the introduction of multiple genes in the vector. For example a 25 kb deletion has been described (Lieber et al. 1996 J. Virol. 70: 8944-8960) and a cloning vector deleted of all viral genes has been reported (Fisher et al 1996 Virology 217: 11-22.) which will permit
20 the introduction of more than 35kb of heterologous DNA. Such vectors may be used to generate an adenoviral primary vector according to the invention encoding two or three transcription units for construction of the retroviral secondary vector.

Embodiments of the invention described solve one of the major problems associated with adenoviral and other viral vectors, namely that gene expression from such vectors is
25 transient. The retroviral particles generated from the primary target cells can infect secondary target cells and gene expression in the secondary target cells is stably maintained because of the integration of the retroviral vector genome into the host cell genome. The secondary target cells do not express significant amounts of viral protein antigens and so are less immunogenic than the cells transduced with adenoviral vector.

The use of a retroviral vector as the secondary vector is also advantageous because it allows a degree of cellular discrimination, for instance by permitting the targeting of rapidly dividing cells. Furthermore, retroviral integration permits the stable expression of therapeutic genes in the target tissue, including stable expression in proliferating target cells.

Preferably, the primary viral vector preferentially infects a certain cell type or cell types. More preferably, the primary vector is a targeted vector, that is it has a tissue tropism which is altered compared to the native virus, so that the vector is targeted to particular cells. The term "targeted vector" is not necessarily linked to the term "target cell". "Target cell" simply refers to a cell which a vector, whether native or targeted, is capable of infecting or transducing.

The preferred, adenoviral primary vector according to the invention is also preferably a targeted vector, in which the tissue tropism of the vector is altered from that of a wild-type adenovirus. Adenoviral vectors can be modified to produce targeted adenoviral vectors for example as described in Krasnykh et al. 1996 J. Virol 70: 6839-6846; Wickham et al 1996 J. Virol 70: 6831-6838; Stevenson et al. 1997 J. Virol. 71: 4782-4790; Wickham et al. 1995 Gene Therapy 2: 750-756; Douglas et al. 1997 Neuromuscul. Disord. 7:284-298; Wickham et al. 1996 Nature Biotechnology 14: 1570-1573.

Primary target cells for the vector system according to the invention include but are not limited to haematopoietic cells (including monocytes, macrophages, lymphocytes, granulocytes or progenitor cells of any of these); endothelial cells; tumour cells; stromal cells; astrocytes or glial cells; muscle cells; and epithelial cells.

Thus, a primary target cell according to the invention, capable of producing the second viral vector, may be of any of the above cell types. In a preferred embodiment, the primary target cell according to the invention is a monocyte or macrophage infected by a defective adenoviral vector containing a first transcription unit for a retroviral gag-pol and a second transcription unit capable of producing a packageable defective retroviral genome. In this

case at least the second transcription unit is preferably under the control of a promoter-enhancer which is preferentially active in a diseased location within the body such as an ischaemic site or the micro-environment of a solid tumour. In a particularly preferred embodiment of this aspect of the invention, the second transcription unit is constructed
5 such that on insertion of the genome into the secondary target cell, an intron is generated which serves to reduce expression of the viral *env* gene and permit efficient expression of a therapeutic gene.

The secondary viral vectors may also be targeted vectors. For retroviral vectors, this may
10 be achieved by modifying the envelope protein. The envelope protein of the retroviral secondary vector needs to be a non-toxic envelope or an envelope which may be produced in non-toxic amounts within the primary target cell, such as for example a MMLV amphotropic envelope or a modified amphotropic envelope. The safety feature in such a case is preferably the deletion of regions or sequence homology between retroviral
15 components.

The secondary target cell population may be the same as the primary target cell population. For example delivery of a primary vector of the invention to tumour cells leads to replication and generation of further vector particles which can transduce further tumour
20 cells. Alternatively, the secondary target cell population may be different from the primary target cell population. In this case the primary target cells serve as an endogenous factory within the body of the treated individual and produce additional vector particles which can infect the secondary target cell population. For example, the primary target cell population may be haematopoietic cells transduced by the primary vector *in vivo* or *ex vivo*. The
25 primary target cells are then delivered to or migrate to a site within the body such as a tumour and produce the secondary vector particles, which are capable of transducing for example tumour cells within a solid tumour.

The invention permits the localised production of high titres of defective retroviral vector
30 particles *in vivo* at or near the site at which action of a therapeutic protein or proteins is required with consequent efficient transduction of secondary target cells. This is more

efficient than using either a defective adenoviral vector or a defective retroviral vector alone.

The invention also permits the production of retroviral vectors such as MMLV-based
5 vectors in non-dividing and slowly-dividing cells *in vivo*. It had previously been possible
to produce MMLV-based retroviral vectors only in rapidly dividing cells such as tissue
culture-adapted cells proliferating *in vitro* or rapidly dividing tumour cells *in vivo*.
Extending the range of cell types capable of producing retroviral vectors is advantageous
for delivery of genes to the cells of solid tumours, many of which are dividing slowly, and
10 for the use of non-dividing cells such as endothelial cells and cells of various
haematopoietic lineages as endogenous factories for the production of therapeutic protein
products.

The delivery of one or more therapeutic genes by a vector system according to the
15 invention may be used alone or in combination with other treatments or components of the
treatment. Diseases which may be treated include, but are not limited to: cancer,
neurological diseases, inherited diseases, heart disease, stroke, arthritis, viral infections and
diseases of the immune system. Suitable therapeutic genes include those coding for
tumour suppressor proteins, enzymes, pro-drug activating enzymes, immunomodulatory
20 molecules, antibodies, engineered immunoglobulin-like molecules, fusion proteins,
hormones, membrane proteins, vasoactive proteins or peptides, cytokines, chemokines,
anti-viral proteins, antisense RNA and ribozymes.

In a preferred embodiment of a method of treatment according to the invention, a gene
25 encoding a pro-drug activating enzyme is delivered to a tumour using the vector system of
the invention and the individual is subsequently treated with an appropriate pro-drug.
Examples of pro-drugs include etoposide phosphate (used with alkaline phosphatase Senter
et al., 1988 Proc. Natl. Acad. Sci. 85: 4842-4846); 5-fluorocytosine (with Cytosine
deaminase Mullen et al. 1994 Cancer Res. 54: 1503-1506); Doxorubicin-N-p-
30 hydroxyphenoxyacetamide (with Penicillin-V-Amidase (Kerr et al. 1990 Cancer Immunol.
Immunother. 31: 202-206); Para-N-bis(2-chloroethyl) aminobenzoyl glutamate (with

Carboxypeptidase G2); Cephalosporin nitrogen mustard carbamates (with b-lactamase); SR4233 (with P450 Reducase); Ganciclovir (with HSV thymidine kinase, Borrelli et al. 1988 Proc . Natl. Acad. Sci. 85: 7572-7576) mustard pro-drugs with nitroreductase (Friedlos et al. 1997J Med Chem 40: 1270-1275) and Cyclophosphamide or Ifosfamide
5 (with a cytochrome P450 Chen et al. 1996 Cancer Res 56: 1331-1340).

Further provided according to the invention are methods of controlling production of a therapeutic gene such that the therapeutic gene is preferentially expressed in the secondary target cell population and is poorly expressed or not expressed at a biologically significant
10 level in the primary target cell.

In accordance with the invention, standard molecular biology techniques may be used which are within the level of skill in the art. Such techniques are fully described in the literature. See for example; Sambrook *et al* (1989) Molecular Cloning; a laboratory
15 manual; Hames and Glover (1985 - 1997) DNA Cloning: a practical approach, Volumes I- IV (second edition); Methods for the engineering of immunoglobulin genes are given in McCafferty *et al* (1996) "Antibody Engineering: A Practical Approach".

In summation, the present invention relates to a novel delivery system suitable for
20 introducing one or more NOIs into a target cell.

In one broad aspect the present invention relates to a retroviral vector comprising a functional splice donor site and a functional splice acceptor site; wherein the functional splice donor site and the functional splice acceptor site flank a first nucleotide sequence
25 of interest ("NOI"); wherein the functional splice donor site is upstream of the functional splice acceptor site; wherein the retroviral vector is derived from a retroviral pro-vector; wherein the retroviral pro-vector comprises a first nucleotide sequence ("NS") capable of yielding the functional splice donor site and a second NS capable of yielding the functional splice acceptor site; wherein the first NS is downstream of the second NS;
30 such that the retroviral vector is formed as a result of reverse transcription of the retroviral pro-vector.

In a further broad aspect, the present invention provides a hybrid viral vector system for *in vivo* gene delivery, which system comprises one or more primary viral vectors which encode a secondary viral vector, the primary vector or vectors capable of infecting a first target cell and of expressing therein the secondary viral vector, which secondary vector is capable of transducing a secondary target cell.

Preferably the primary vector is obtainable from or is based on a adenoviral vector and/or the secondary viral vector is obtainable from or is based on a retroviral vector preferably a lentiviral vector.

The invention will now be further described by way of example in which reference is made to the following Figures:

Figure 1 which shows the structure of a retroviral proviral genome;

Figure 2 which shows the addition of a small T splice donor pLTR_κ;

Figure 3 which shows a diagrammatic representation of pL-SA-N_κ;

Figure 4 which shows a diagrammatic representation of pL-SA-N with a splice donor deletion;

Figure 5 which shows the sequence of MLV pICUT_κ;
(SEQ ID NO:1)

Figure 6 which shows the insertion of a splice donor at CMV/R junction of EIAV LTR plasmid;

Figure 7 which shows the insertion of a splice acceptor into pEGASUS-1_κ;

Figure 8 which shows the removal of a wild-type splice donor from EIAV vector;

Figure 9 which shows the combination of pCMVLTR+SD with pEGASUS +SA (noSD) to create pEICUT-1;

5 Figure 10 which shows the construction of pEICUT-LacZ;

B Figure 11 which shows the pEICUT-LacZ sequence; ^(SEQ ID NO:2)

^{mm}
4.20.02
Figures 12A and 12B

Figure 12 which shows the vector configuration in both transfected and transduced cells;

^{mm}
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10 Figures 13A and 13B

Figure 13 which shows the restriction of gene expression to either packaging or transduced cells;

^{mm}
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Figures 14A-14C

15 Figure 14 which shows the construction of a MLV pICUT Neo-p450 vector that restricts hygromycin expression to producer cells and 2B6 (a p450 isoform) expression to transduced cells;

B Figure 15 which shows a sequence comparison of mutant *env* (m4070A) ^(SEQ ID NO:5) with wild type
B MMLV sequence ^(SEQ ID NO:4) from the 3' end of the *pol* gene;

20 B Figure 16 which shows the complete sequence ^(SEQ ID NO:3) of the modified *env* gene m4070A;

Figure 17 which shows a restricted gene expression construct; 4070A Envelope to a first cell; p450 to a second cell;

25 Figure 18 which shows the use of an intron to restrict NOI (in this example p450) expression to a transduced cell;

Figure 19 which shows a pictorial representation of the Transfer vector-pE1sp1A;

30 Figure 20 which shows a pictorial representation of pE1sp1A construct;

Figure 21 which shows a pictorial representation of pE1RevE construct;

Figure 22 which shows a pictorial representation of pE1HORSE3.1- *gagpol* construct;

Figure 23 which shows a pictorial representation of pE1PEGASUS4-Genome construct;

Figure 24 which shows a pictorial representation of pCI-*Neo* construct;

Figure 25 which shows a pictorial representation of pCI-Rab construct;

Figure 26 which shows a pictorial representation of pE1Rab construct;

Figure 27a is a schematic representation of the natural splicing configuration in a retroviral vector;

Figure 27b is a schematic representation of the splicing configuration in known retroviral vectors;

Figure 27c is a schematic representation of the splicing configuration according to the present invention; and

Figure 28 is a schematic representation of the dual hybrid viral vector system according to the present invention.

In slightly more detail:

Figure 1 shows the structure of a retroviral proviral genome. In this regard, the simplest retroviruses such as the murine oncoretroviruses have three open reading frames; *gag*, *pol* and *env*. Frameshift during *gag* translation leads to *pol* translation. Env expression and translation is achieved by splicing between the splice donor (SD) and splice acceptor

(SA) shown. The packaging signal is indicated as *Psi* and is only contained in the full length transcripts - not the *env* expressing sub-genomic transcripts where this signal is removed during the splicing event.

5 Figure 2 schematically shows the addition of small T splice donor to pLTR. Here, the small-t splice donor sequence is inserted into an LTR vector downstream of the start of transcription but upstream of *R* sequence such that upon reverse transcription (in the final construct) the U3-splice donor-R cassette is 'inherited' to 5' end of the proviral vector and RNA transcripts expressed contain a splice donor sequence near their 5'
10 terminus.

Figure 3 shows a schematic diagram of pL-SA-N. Both the consensus splice acceptor (T/C)nNC/TAG-G (Mount 1982 Nucleic Acids Res 10: 459-472) and branch point are shown in underline and bold.. The arrow indicates the intron/exon junction. Here, the
15 consensus splice acceptor sequence is inserted into the *Stu1/BamH1* sites of pLXSN. By such positioning this acceptor will therefore interact with any upstream splice donor (in the final RNA transcripts).

Figure 4 shows a schematic diagram for the construction of pL-SA-N with a splice donor deletion. The gT to gC change is made by performing a PCR reaction on the pL-SA-N
20 vector with the two oligonucleotides shown below. The resulting product is then cloned *Spe1-Asc1* into pL-SA-N thus replacing the wild-type splice donor gT with gC. Both *Spe1* and *Asc1* sites are shown in bold and the mutation in the *Spe1* oligonucleotide shown in capital bold.

25 Figure 5 shows the sequence of MLV pICUT.

Figure 6 shows a schematic diagram of the insertion of splice donor at CMV/R junction of ELAV LTR plasmid. PCR is performed with the two oligonucleotides outlined below
30 and the resulting PCR product cloned *Sac1-BamH1* into CMVLTR with the equivalent piece removed. In the *Sac1* oligonucleotide the arrow indicates the start of transcription,

the new insert is shown in capital with splice donor sequence underlined. The start of R is shown in italics.

Figure 7 shows a schematic diagram of the insertion of splice acceptor into pEGASUS-1.

5 Here, the double stranded oligonucleotide described below is inserted into *Xho*1-*Bpu*1102 digested pEGASUS-1 to generate plasmid pEGASUS+SA. Both consensus splice acceptor (T/C)nNC/TAG-G (Mount 1982 *ibid*) and branch point are shown in underline and bold. The arrow indicates the intron/exon junction.

10 Figure 8 shows a schematic diagram of the removal of wild-type splice donor from ELAV vector. Splice donor sequence removed by overlapping PCR using the oligonucleotides described below and the template pEGASUS+SA. First separate PCR reactions are performed with oligos1+2 and oligos3+4. The resulting amplified products are then eluted and used combined in a third PCR reaction. After 10 cycles of
15 this third reaction oligo2 and 4 are then added. The resulting product is then cloned *Sac*1-*Sal*1 into pEGASUS+SA to create the plasmid pEGASUS+SA(noSD). The position of the splice donor (SD) is indicated. The point mutation changing the wild-type splice donor from GT to GC is shown in bold both in oligo1 and the complementary oligo3.

20 Figure 9 shows a schematic diagram of combining pCMVLTR+SD with pEGASUS+SA(noSD) to create pEICUT-1. Here, one inserts the *Mlu*1 fragment of pEGASUS+SA(noSD) into the unique *Mlu*1 site of pCMV-LTR.

25 Figure 10 shows a schematic diagram of the construction of pEICUT-LacZ. It is made by the insertion of the *Xho*1-*Bpu*1102 LacZ fragment from pEGASUS-1 and inserting it into the *Xho*1-*Bpu*1102 site of pEICUT-1 as outlined below.

Figure 11 shows the pEICUT-LacZ sequence.

30 Figure 12 shows a schematic diagram of the vector configuration in both transfected and transduced cells. Here, the starting pICUT vector contains no splice donor upstream of

a splice acceptor (in this instance the consensus splice acceptor derived from IgSA) and therefore the resulting RNA transcripts will not be spliced. Thus all transcripts will be full length transcripts containing a packaging signal (A). Upon transduction however the splice donor (in this instance the small-T spliced donor) is 'inherited' to the 5' of the proviral vector such that all RNA transcripts now produced contain splice donor upstream of a splice acceptor i.e. an intron and thus maximal splicing achieved (B).

Figure 13 shows a schematic diagram of the restriction of gene expression to either packaging and transduced cells. Restriction of gene expression in this instance is achieved by placing the hygromycin ORF upstream of the neomycin ORF in MLV pEICUT (a). By this cloning strategy the resulting vector will now express RNA transcripts that express hygromycin only in transfected cells because ribosome 5' cap-dependent translation will read only the upstream ORF efficiently. However upon transduction hygromycin is now contained within a functional intron and is thus deleted from mature transcripts (b) and thus neomycin ORF is now translated in a 5' cap-dependent manner.

Figure 14 shows a schematic diagram of the construction of a MLV pICUT *Neo*-p450 vector that restricts hygromycin expression to producer cells and 2B6 (a p450 isoform) expression to transduced cells. The starting vector for this construction is the pICUT vector of Figure 13 containing both *hygro* and *neo*. The *neo* gene is replaced with the complete p450 2B6 cDNA as follows: The complete 2B6 cDNA is obtained by RT-PCR on human liver RNA (Clontech) using the following primers:

25 5'ttcgatgatcaccaccatggaactcagcgctcctcctcttcctgcac3'

5'ttcgagccggctcatcagcggggcaggaagcggatctggtatgttg3'

This generates the complete 2B6 cDNA with an optimised kosak sequence flanked with unique *Bcl*I and *Ngo*M1 sites. This cDNA is then cloned into the *Bcl*I-*Ngo*M1 site of

pICUT-Hyg-Neo thus replacing *Neo* with p450 (see (A) below). Also shown below are the proviral DNA constructs in both transfected (B) and transduced (C) cells.

Figure 15 is a sequence comparison of mutant env (m4070A) with wild type MMLV sequence from the 3' end of the pol gene.

Figure 16 is the complete sequence of altered 4070A.

Figure 17 shows a gene restricted expression retroviral vector whereby the first NOI (the 4070A envelope ORF) is expressed in the initial vector and the second NOI (in this instance p450) is expressed only after vector replication. After replication the 4070A gene is located within a functional intron and thus removed during RNA splicing.

Figure 18 shows a retroviral expression vector whereby the 5' end of the p450 gene (flush to a splice donor) is only found upstream of the 3' end of the p450 gene (flush to SA) after replication and thus only after replication is a functional p450 gene expressed (from spliced mRNA).

EXAMPLES

Example 1 Construction of a split-intron MLV vector .

(i) Addition of small-T splice donor:

The starting plasmid for this construct is pLXSN (Miller *et al* 1989 *ibid*); Firstly this construct is digested with *Nhe*1 and the backbone re-ligated to create an LTR (U3-R-U5) plasmid. Into this plasmid is then inserted an oligonucleotide containing the splice donor sequence between the *Kpn*1-*Bbe*1 sites. Also contained within this oligonucleotide, downstream of the splice donor is the MLV R sequence up to the *Kpn*1. The resulting plasmid is named 3'LTR-SD (see Figure 2).

(ii) Addition of splice acceptor:

The splice acceptor sequence used in this construct (including the branch point- an A residue between 20 and 40 bases upstream of the splice acceptor involved in intron lariat formation (Aebi *et al* 1987 Trends in Genetics 3: 102-107) is derived from an immunoglobulin heavy chain variable region mRNA (Bothwell *et al* 1981 Cell 24: 625-637) but with a consensus/optimised acceptor site. Such a sequence signal is also present in pCI (Promega). This acceptor sequence is firstly inserted into the *Bam*H1-*Stu*1 sites of pLXSN as double stranded oligonucleotide to create the vector pL-SA-N (note: SV40 promoter is lost from pLNSX during cloning). See Figure 3 for an outline of the cloning strategy.

(iii) Removal of original splice donor from pL-SA-N.

The removal of the splice donor contained within the *gag* sequence of pL-SA-N is achieved by PCR based site directed mutagenesis. Two oligonucleotides are used to PCR amplify the region spanning the *Asc*1 and *Spe*1 unique sites of pL-SA-N. Also incorporated in the *Spe*1-spanning oligonucleotide is the agGTAag to agGCAag change also found in the splicing negative pBABE vectors (Morgenstern *et al* 1990 *ibid*). See Figure 4 for cloning strategy outline.

(iv) Combining pL(noSD)-SA-N with 3'LTR-SD.

The pL(noSD)SA-N plasmid contains a normal MLV derived 3'LTR. This is replaced with the 3'LTR-SD sequence by taking the *Nhe*1 insert from pL(noSD)SA-N and dropping it into the *Nhe*1 digested 3'LTR-SD vector. The resulting plasmid, named pICUT (Intron Created Upon Transduction) contains all the features of this new generation of retroviral vector (see Figure 5 for sequence data)

Example 2 Construction of a split-intron Lentivector.

Construction of initial EIAV lentiviral expression vector (also see patent application GB 9727135.7)

5

For the construction of a split-function lentiviral vector the starting point is the vector named pEGASUS-1 (see patent application GB 9727135.7). This vector is derived from infectious proviral EIAV clone pSPEIAV19 (accession number: U01866; Payne *et al* 1994). Its construction is outlined as follows: First; the EIAV LTR, amplified by PCR, is cloned into pBluescript II KS+ (Stratagene). The *MluI/MluI* (216/8124) fragment of pSPEIAV19 is then inserted to generate a wild-type proviral clone (pONY2) in pBluescript II KS+ (Figure 1). The *env* region is then deleted by removal of the *Hind* III/*Hind* III fragment to generate pONY2-H. In addition, a *BglIII/NcoI* fragment within *pol* (1901/4949) is deleted and a β -galactosidase gene driven by the HCMV IE enhancer/promoter inserted in its place. This is designated pONY2.10nlsLacZ. To reduce EIAV sequence to 759 base pairs and to drive primary transcript off a CMV promoter: First; sequence encompassing the EIAV polypurine tract (PPT) and the 3'LTR are PCR amplified from pONY2.10LacZ using primers:

20 PPTEIAV+ (Y8198): GACTACGACTAGTGTATGTTTAGAAAAACAAGG,
and

3'NEG*SpeI*(Y8199): CTAGGCTACTAGTACTGTAGGATCTCGAACAG.

25 The PCR product is then cloned into the *SpeI* site of pBS II KS⁺; orientated such that U5 is proximal to *NotI* in the pBlueScript II KS⁺

Next, for the reporter gene cassette, a CMV promoter/*LacZ* from pONY 2.10nlsLacZ is removed by *PstI* digest and cloned into the *PstI* site of pBS.3'LTR orientated such that *LacZ* gene is proximal to the 3'LTR, this vector is named pBS CMVLacZ.3'LTR.

30

The 5' region of the EIAV vector is constructed in the expression vector pCIE_{neo} which is derivative of pCIneo (Promega)-modified by the inclusion of approximately 400 base pairs derived from the 5' end of the full CMV promoter as defined previously. This 400 base pair fragment is obtained by PCR amplification using primers:

VSAT1: (GGGCTATATGAGATCTTGAATAATAAAATGTGT) and

VSAT2: (TATTAATAACTAGT) and

- 10 pHIT60 (Soneoka *et al* 1995 Nucleic Acids Res 23: 628-633) as template. The product is digested with *Bgl*III and *Spe*I and cloned into the *Bgl*III/*Spe*I sites of pCIE-Neo.

A fragment of the EIAV genome running from the R region to nt 150 of the *gag* coding region (nt 268 to 675) is amplified from pSEIAV with primers:

15

B CMV5'EIAV2: (SEQ ID NO: 7)

(Z0591)(GCTACGCAGAGCTCGTTTAGTGAACCGGGCACTCAGATTCTG:

B

(sequences underlined anneals to the EIAV R region) and (SEQ ID NO: 8)

20

3'PSI.NEG (GCTGAGCTCTAGAGTCCTTTTCTTTTACAAAGTTGG).

- The resulting PCR product is flanked by *Xba*I and *Sac*I sites. This is then cut and cloned into the pCIE-Neo *Xba*I-*Sac*I sites. The resulting plasmid, termed
- 25 pCIE_{neo}5'EIAV now contains the start of the EIAV R region at the transcriptional start point of the CMV promoter. The CMV*LacZ*/3LTR cassette is then inserted into the pCIE_{neo}5'EIAV plasmid by taking the *Apa*I to *Not*I fragment from pBS.CMV*LacZ*.3LTR and cloning it into the *Sal*I-*Not*I digested pCIE_{neo}.5'EIAV (the *Sal*I and *Apa*I sites is T4 "polished" to create blunt the ends prior to the vector and
- 30 insert respective *Not*I digests). The resulting plasmid is named pEGASUS-1.

For use as a gene delivery vector pEGASUS-1 requires both *gag/pol* and *env* expression provided *in trans* by a packaging cell. For the source of *gag/pol* an EIAV *gagpol* expression plasmid (pONY3) is made by inserting the *Mlu* I/*Mlu* I fragment from pONY2-H into the mammalian expression plasmid pCI-*neo* (Promega) such that the *gag-pol* gene is expressed from the hCMV-MIE promoter-enhancer and contains no LTR sequences. For the source of *env*; the pRV583 VSV-G expression plasmid is routinely used. These three vectors are used in a three plasmid co-transfection as described for MLV-based vectors (Soneoka *et al* 1995 Nucl. Acids Res. 23:628-633) the resulting virus routinely titres at between 10^4 and 10^5 *lacZ* forming units per ml on D17 fibroblasts.

Construction of a EIAV lentiviral version vector of pICUT; named pEICUT

To construct pEICUT firstly pEGASUS-1 the *Xma*I-*SexA*I fragment is removed from pEGASUS-1 and the ends 'blunted' with T4 polymerase and plasmid re-ligated to create a plasmid containing only the CMV-R-U5 part of pEGASUS-1 which retains the SV40-*Neo* cassette in the backbone. This plasmid is named CMVLTR. To insert a splice donor at the CMV-R border PCR is carried out with the two oligonucleotides shown below in Figure 6 and as outlined in the Figure 6 legend. The resulting plasmid is named pCMVLTR+SD. The same immunoglobulin based consensus splice acceptor as for MLV pICUT (see earlier) is used in the EIAV version. This is inserted using oligonucleotides described in Figure 7 into the *Xho*I-*Bpu*1102 site of pEGASUS-1 to create the plasmid pEGASUS+SA. The wild-type splice donor of EIAV is removed by carrying out overlapping PCR with the oligonucleotides and methodology as described in Figure 8, using pEGASUS+SA as a template to generate the plasmid pEGASUS+SA(noSD). To then create pEICUT-1, the *Mlu*I-*Mlu*I fragment from pEGASUS+SA(noSD) is then inserted into the unique *Mlu*I site of pCMVLTR+SD to generate pEICUT-1 (see Figure 9). *LacZ* can be then transferred from pEGASUS-1 into pEICUT-1 by *Xho*I-*Bpu*1102 digest and insertion to create pEICUT-Z (see Figure 10; for sequence data see Figure 11).

Both the MLV and ELAV pICUT vectors contain a strong splice acceptor upstream of the splice donor and therefore no functional intron (introns require splice donors positioned 5' of splice acceptors). For this reason, when the vector is transfected into producer cells the resulting transcripts generated will not be spliced. Thus the packaging signal
5 will not be lost and as a consequence maximal packaging is achievable (see Figure 12).

However because of the unique way by which retroviruses replicate, upon transduction, transcripts generated from the integrated pICUT vector will differ from those of transfected cells described above. This is because during replication the 3'U3 promoter
10 (up to the 5' start of R) is copied and used as the 5' promoter in transduced cells. For this reason transcripts generated from integrated pICUT will now contain a strong splice donor 5' of a strong splice acceptor, both of which being located upstream of the *neo* ORF. Such transcripts will therefore contain a functional intron in the 5'UTR (untranslated region) and thus be maximally spliced and translated.

15 Another advantage of such vectors described above is that because the intron is created only upon transduction it is possible to limit gene expression to either packaged or transduced cells. One example of how this is achieved is outlined in Figures 13. The strategy entails the cloning of a second gene (in this example hygromycin) upstream of
20 the splice acceptor. This is achieved by taking out the hygromycin cDNA on a *SalI* fragment from SelctaVector Hygro (Ingenius; Oxfordshire, UK), and cloning this into a *XhoI* site (located upstream of the splice acceptor) of pICUT. This vector selectively expresses hygromycin in the transfected cells and neomycin in transduced cells. The reason for this is that in any one mRNA transcript only the first gene is translated by the
25 ribosome without the aid of internal ribosome binding sites (IRESs). In the transfected cell this gene will be hygromycin. However in the transduced cells because the hygromycin open reading frame (ORF) is contained within a functional intron this gene will now be removed from mature mRNA transcripts thus allowing *neo* ORF translation.

30 Vectors with such cell specific gene expression may be of clinical use for a variety of reasons; By way of example, expression of resistance markers can be restricted to

producer cells- where they are required and not in transduced cells where they may be immunogenic. By way of another example, expression of toxic genes such as ricin and dominant negative signalling proteins could be restricted to transduced cells where they may be required to optionally arrest cell growth or kill cells but not in producer cells-
5 where such features would prevent high titre virus production. Figure 14 shows a *Neo*-p450 MLV pICUT construct such that only *Neo* is expressed in producer cells and the pro-drug p450 2B6 isoform expressed in transduced cells.

Another benefit of creating an intron upon transduction is that any essential elements
10 required for vector function can now be placed inside a functional intron, which is created upon transduction, and be removed from transduced cell transcripts. By way of example, with both the MLV and the lentivector pICUT vectors, the viral transcript contained the functional *Psi* packaging signal (see Bender *et al* 1987 for the position of *Psi* in MLV; see patent application GB 9727135.7 for position of *Psi* in EIAV) within an
15 intron which was created upon transduction and removed from the transduced cell transcripts.

The benefits from such an arrangement include:

20 (i) Enhanced translation from resulting transcripts because ribosomes may "stutter" in the presence of a *Psi* secondary structure- if present (Krall *et al* 1996 *ibid* and reference therein).

(ii) In the absence of the packaging signal, transcript packaging by endogenous
25 retroviruses is prevented.

(iii) Unwanted premature translation initiation is prevented when viral essential elements such as *gag* (and other potential ATG translation start sites) are removed from the transcripts expressed in transduced cells. This is of particular benefit when packaging
30 signals extend into *gag* as is the case for both the EIAV and MLV pICUT vectors.

(iv) Promoter, enhancers and suppressors may be placed within an intron created upon transduction thus mimicking other transcript arrangements like those generated from CMV that contain such entities within introns (Chapman *et al* 1991 *ibid*)

5 In summation the novel pICUT vector system described in the present invention facilitates the following arrangements:

(I) Maximal packaging and reduced translation of transcripts in producer cells.

10 (ii) Maximal splicing and therefore intron enhanced translation of transcripts in transduced cells

(iii) Restriction of gene and/or viral essential element expression to either producer or transduced cells.

15

Example 3 Construction of an MMLV amphotropic *env* gene with minimal homology to the *pol* gene and a *gag-pol* transcription cassette

20 In the Moloney murine leukaemia virus (MMLV), the first approximately 60 bps of the *env* coding sequence overlap with sequences at the 3' end of the *pol* gene. The region of homology between these two genes was removed to prevent the possibility of recombination between them in cells expressing both genes.

25 The DNA sequence of the first 60 bps of the coding sequence of *env* was changed while retaining the amino acid sequence of the encoded protein as follows. A synthetic oligonucleotide was constructed to alter the codon usage of the 5'-end of *env* (See Figure 15) and inserted into the remainder of *env* as follows.

30 The starting plasmid for re-construction of the 5' end of the 4070A gene was the pCI plasmid (Promega) into which had previously been cloned the *Xba*I-*Xba*I fragment containing the 4070A gene from pHIT456 (Soneoka *et al* 1995 *ibid*) to form pCI-4070A.

A PCR reaction was performed with primers A and B (Figure 15) on pCI-4070A to produce a 600 base pair product. This product was then cloned between the *Nhe*I and *Xho*I sites of pCI-4070A. The resulting construct was sequenced across the *Nhe*I/*Xho*I region. Although the amino acid sequence of the resulting gene is the same as the original 4070A, the region of homology with the *pol* gene is removed.

The complete sequence of the modified *env* gene m4070A is given in Figure 16. This sequence is inserted into the expression vector pCI (Promega) by standard techniques. The CMV *gag-pol* transcription unit is obtained from pHIT60 (Soneoka *et al* 1995 *ibid*).

Example 4 Deletion of *gag* sequences from the retroviral packaging signal.

A DNA fragment containing the LTR and minimal functional packaging signal is obtained from the retroviral vector MFG (Bandara *et al* 1993 Proc Natl Acad Sci 90: 10764-10768) or MMLV proviral DNA by PCR reaction using the following oligonucleotide primers:

B ^(SEQ ID NO:9)
HindIII: GCATTAAAGCTTTGCTCT

B 20 ^(SEQ ID NO:10)
L523: GCCTCGAGCAAAAATTCAGACGGA

This PCR fragment contains MMLV nucleotides +1 to +523 and thus does not contain *gag* coding sequences which start at +621 (numbering based on the nucleotide sequence of MMLV Shinnick *et al* 1981 Nature 293: 543-548).

25 The PCR fragment can be used to construct a retroviral genome vector by digestion using *Hind*III and *Xho*I restriction enzymes and sub-cloning using standard techniques. Such vectors contain no homology with *gag* coding sequences.

Example 5 Construction of defective retroviral genome

The transcription unit capable of producing a defective retroviral genome is shown in Figure 17. It contains the following elements: a hypoxia regulated promoter enhancer comprising 3 copies of the PGK - gene HRE and a SV40 promoter deleted of the 72bp-repeat enhancer from pGL3 (Promega); a MMLV sequence containing R, U5 and the packaging signal; the coding sequence of m4070A (Example 3); a splice acceptor; a cloning site for insertion of a coding sequence for a therapeutic protein; the polypyrimidine tract from MMLV; a second copy of the HRE-containing promoter-enhancer; a splice donor site; and a second copy of R, U5.

- On reverse transcription and integration of the vector into the secondary target cell, the splice donor is introduced upstream of the *env* gene causing it to be removed from mRNA by splicing and thereby permitting efficient expression of the therapeutic gene only in the secondary target cell (See Figure 17).

Example 6 Construction of a conditional expression vector for Cytochrome P450

Figure 18 shows the structure of retroviral expression vector cDNA coding sequences from the cytochrome P450 gene in two halves such that only upon transduction is the correct splicing achieved to allow P450 expression. This therefore restricts expression to transduced cells.

- 1) The starting plasmid for the construction of this vector is pLNSX (Miller and Rosman 1989 BioTechniques 7: 980-990). The natural splice donor (...agGTAag...) contained within the packaging signal of pLNSX (position 781/782) is mutated by PCR mutagenesis using the ALTERED SITES II mutagenesis kit (Promega) and a synthetic oligonucleotide of the sequence:

5'-caaccaccgggagGCaagctggccagcaactta-3'

- 2) A CMV promoter from the pCI expression vector (Promega) is isolated by PCR using the following two oligonucleotides:

(SEQ ID NO: 12)
 B Primer 1: 5'-atcggctagcagatcttcaatattggccattagccatat-3'

(SEQ ID NO: 13)
 B Primer 2: 5'-atcgagatctgcggccgcttacctgccagtcctcacgaccaa-3'

5

This produces a fragment containing the CMV promoter with a 5' *Nhe*1 site (Primer 1) and a 3' *Not*1 and *Xba*1 site (Primer 2). It is cut with *Nhe*1 and *Xba*1 and cloned into pLNSX from which an *Nhe*1-*Nhe*1 fragment has been removed.

10 3) The 5' end of a cytochrome P450 cDNA coding sequence is isolated by RT-PCR from human liver RNA (Clontech) with the following primers:

(SEQ ID NO: 14)
 B Primer 3: 5'-atcggcgccgcccaccatggaactcagcgtcctcctcttccttgaccctagg-3'

(SEQ ID NO: 15)
 B 15 Primer 4: 5'-atcggcgccgcacttacCtgtgtgccccaggaaagtatttcaagaagccag-3'

This amplifies the 5' end of the p450 from the ATG to residue 693 (numbering from the translation initiation site Yamano *et al* 1989 Biochem 28:7340-7348). Contained on the 5' end of the fragment (derived from Primer 3) is also a *Not*1 site and an optimised
 20 "Kozak" translation initiation signal. Contained on the 3' end of the sequence (derived from primer 4) is another *Not*1 site and a consensus splice donor sequence (also found in pCI and originally derived from the human beta globin gene) with the GT splice donor pair located flush against residue 704 of P450 (the complementary residue is shown in uppercase in Primer 4). This fragment is digested with *Not*1 and cloned into the *Not*1
 25 digested plasmid generated in step 2.

4) The *Nhe*1-*Nhe*1 fragment removed during the cloning of step 2 is then re-introduced into the plasmid of step 3. This creates a retroviral vector as described in Figure 17 but missing the 3' end P450.

30

5) The 3' of the P450 coding sequence is isolated by RT-PCR amplification from human liver RNA (Clontech) using the following primers:

B Primer 5: ^(SEQ ID NO: 16)
actgtgatcataggcacctattggtcttactgacatccactttctctccacagGcaagttacaaaacctgc
5 aggaaatcaatgcttacatt-3'

B Primer 6: ^(SEQ ID NO: 17)
actgatcgattccctcagccccttcagcggggcaggaagc-3'

This generates the PCR amplified 3' end of P450 from residue 705 (in uppercase primer
10 5) and extends past the translation termination codon. Contained within the 5' end of
this product and generated by primer 5 is a *Bcl*I restriction site and a consensus splice
acceptor and branch point (also found in pCI and originally from an immunoglobulin
gene) upstream of residue 705. Contained at the 3' end of this product downstream of
the stop codon and generated by primer 6 is a *Cla*I site. This PCR product is then
15 digested with *Bcl*I and *Cla*I and cloned into the vector of step 3 with the *Bcl*I-*Cla*I
fragment removed to generate the retroviral vector as shown in Figure 18.

The following examples describe the construction of an adenolentiviral system that can be
used for the transient production of lentivirus *in vitro* or *in vivo*.

20

First Generation Recombinant Adenovirus

The first generation adenovirus vectors consist of a deletion of the E1 and E3 regions of the
virus allowing insertion of foreign DNA, usually into the left arm of the virus adjacent to
25 the left Inverted Terminal Repeat (ITR). The viral packaging signal (194-358 nt) overlaps
with the E1a enhancer and hence is present in most E1 deleted vectors. This sequence can
be translocated to the right end of the viral genome (Hearing & Shenk, 1983 Cell 33: 59-
74). Therefore, in an E1 deleted vector 3.2 kb can be deleted (358-3525 nt).

30 Adenovirus is able to package 105% length of the genome, thus allowing for addition of an
extra 2.1 kb. Therefore, in an E1/E3 deleted viral vector the cloning capacity becomes 7-8
kb (2.1 kb + 1.9 kb (removal of E3) + 3.2 kb (removal of E1). Since the recombinant

adenovirus lacks the essential E1 early gene it is unable to replicate in non-E1 complementing cell lines. The 293 cell line was developed by Graham *et al.* (1977 J Gen Virol 36: 59-74) and contains approximately 4 kb from the left end of the Ad5 genome including the ITR, packaging signal, E1a, E1b and pIX. The cells stably express E1a and E1b gene products, but not the late protein IX, even though pIX sequences are within E1b. In non-complementing cells the E1 deleted virus transduces the cell and is transported to the nucleus but there is no expression from the E1 deleted genome.

First Generation Adenovirus Production System

10 Microbix Biosystems – nbl Gene Sciences

The diagram in Figure 19 shows the general strategy used to create recombinant adenoviruses using the microbix system

- 15 The general strategy involves cloning the foreign DNA into an E1 shuttle vector, where the E1 region from 402-3328 bp is replaced by the foreign DNA cassette. The recombinant plasmid is then co-transfected into 293 cells with the pJM17 plasmid. pJM17 contains a deletion of the E3 region and an insertion of the prokaryotic pBRX vector (including the ampicillin resistance and bacterial ori sequences) into the E1 region at 3.7 map units. This
- 20 40 kb plasmid is therefore too large to be packaged into adeno nucleocapsids but can be propagated in bacteria. Intracellular recombination in 293 cells results in replacement of the amp^r and ori sequences with the insert of foreign DNA.

Example 7 Construction of Transfer plasmids for the creation of Adenoviruses 25 containing EIAV Components

- In order to produce lentiviral vectors four adenovirus need to be made: genome, *gagpol*, *envelope* (rabies G) and *Rev*. The lentiviral components are expressed from heterologous promoters they contain introns where needed (for high expression of *gagpol*, *Rev* and
- 30 Rabies *envelope*) and a polyadenylation signal. When these four viruses are transduced into E1a minus cells the adenoviral components will not be expressed but the heterologous

promoters will allow the expression of the lentiviral components. An example is outlined below (example 1) of the construction of an ELAV adenoviral system (Application number: 9727135.7). The ELAV is based on a minimal system that is one lacking any of the non-essential ELAV encoded proteins (S2, Tat or envelope). The envelope used to pseudotype the ELAV is the rabies envelope (G protein). This has been shown to pseudotype ELAV well (Application number: 9811152.9).

Transfer Plasmids

Described below is the construction of the transfer plasmids containing the ELAV components. The transfer plasmid is pE1sp1A (Figure 20).

The recombinant transfer plasmids can be used to make recombinant adenoviruses by homologous recombination in 293 cells.

A pictorial representation of the following plasmids is attached.

A) pE1RevE – Rev Construct

The plasmid pCI-Rev is cut with *Apa* I and *Cla* I. The 2.3 kb band encoding ELAV Rev is blunt ended with Klenow polymerase and inserted into the *Eco* RV site of pE1sp1A to give plasmid pE1RevE (Figure 21).

B) pE1HORSE3.1 – *gagpol* Construct

pHORSE3.1 was cut with *Sna* BI and *Not* I. The 6.1 kb band encoding ELAV *gagpol* was inserted into pE1RevE cut with *Sna* BI and *Not* I (7.5 kb band was purified). This gives plasmid pE1HORSE3.1 (Figure 22).

C) pE1PEGASUS – Genome Construct

pEGASUS4 was cut with *Bgl* II and *Not* I. The 6.8 kb band containing the EIAV vector genome was inserted into pE1RevE cut with *Bgl* II and *Not* I (6.7kb band was purified).

5 This gave plasmid pE1PEGASUS (Figure 23).

D) pCI-Rab – Rabies Construct

10 In order to make pE1Rab the rabies open reading frame was inserted into pCI-*Neo* (Figure 24) by cutting plasmid pSA91RbG with *Nsi* I and *Ahd* I. The 1.25 kb band was bluntended with T4 DNA polymerase and inserted into pCI-*Neo* cut with *Sma* I. This gave plasmid pCI-Rab (Figure 25).

F) pE1Rab – Rabies Construct

15

pCI-Rab was cut with *Sna* BI and *Not* I. The 1.9 b band encoding Rabies envelope was inserted into pE1RevE cut with *Sna* BI and *Not* I (7.5 b band was purified). This gave plasmid pE1Rab (Figure 26).

20 SUMMARY

The present invention relates to a novel delivery system suitable for introducing one or more NOIs into a target cell.

25 In one preferred aspect the present invention covers a retroviral vector comprising a functional splice donor site and a functional splice acceptor site; wherein the functional splice donor site and the functional splice acceptor site flank a first nucleotide sequence of interest ("NOI"); wherein the functional splice donor site is upstream of the functional splice acceptor site; wherein the retroviral vector is derived from a retroviral pro-vector;
30 wherein the retroviral pro-vector comprises a first nucleotide sequence ("NS") capable of yielding the functional splice donor site and a second NS capable of yielding the functional splice acceptor site; wherein the first NS is downstream of the second NS;

such that the retroviral vector is formed as a result of reverse transcription of the retroviral pro-vector.

Alternatively expressed, this aspect covers a novel delivery system which comprises one or more NOIs flanked by a functional SD and SA provided that this has been generated from a pro-vector in which the order of the SD and SA is reversed to render the splicing non-functional.

This aspect of the present invention can be called the "split-intron" aspect. A schematic diagram showing this aspect of the present invention is provided in Figure 27c. In contrast, Figures 27a and 27b show splicing configurations in known retroviral vectors.

Another broad aspects of the present invention include a novel delivery system which comprises one or more adenoviral vector components capable of packaging one or more lentiviral vector components, wherein optionally the lentiviral vector comprises a split intron configuration.

This aspect of the present invention in the general sense can be called a hybrid viral vector system. In this particular case, the combination of an adenoviral component and a lentiviral component can be called a dual hybrid viral vector system.

A schematic diagram showing this aspect of the present invention is provided in Figure 28.

These and other broad aspects of the present invention are discussed herein.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as

claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

SEQUENCE LISTINGS

SEQ ID NO. 1

5 GCTAGCTTAAGTAACGCCACTTTTGCAAGGCATGGAAAAATACATAACTGAGAATAGAAAAGTTCAGATCAAGG
TCAGGAACAAAGAAACAGCTGAATACCAAACAGGATATCTGTGGTAAGCGGTTCTGCCCCGGCTCAGGGCCA
AGAACAGATGAGACAGCTGAGTGATGGGCCAAACAGGATATCTGTGGTAAGCAGTTCTGCCCCGGCTCAGGGG
CCAAGAACAGATGGTCCCCAGATGCGGTCCAGCCCTCAGCAGTTTCTAGTGAATCATCAGATGTTTCCAGGGT
10 GCCCCAAGGACCTGAAAATGACCCTGTACCTTATTTGAACTAACCAATCAGTTCGCTTCTCGCTTCTGTTCCG
GCGCTTCCGCTCTCCGAGCTCAATAAAAGAGCCACAACCCCTCACTCGGCGCGCCAGTCTTCCGATAGACTG
CGTCGCCCCGGGTACCCGTATTCCCAATAAAGCCTCTTGCTGTTTGCATCCGAATCGTGGTCTCGCTGTTCTT
GGGAGGGTCTCCTCTGAGTGATTGACTACCCACGACGGGGGTCTTTCATTTGGGGGCTCGTCCGGGATTTGGA
GACCCCTGCCCAGGGACCACCGACCCACCACGGGAGGCAAGCTGGCCAGCAACTTATCTGTGTCTGTCCGAT
15 TGTCTAGTGTCTATGTTTGTATGCTTGTGCTGCTGTACTAGTTAGCTAAGTCTGTATCTGGCGGA
CCCGTGGTGGAACTGACGAGTTCTGAACACCCGGCCGCAACCCTGGGAGACGTCCCAGGGACTTTGGGGCCG
TTTTTGTGGCCCGACCTGAGGAAGGGAGTGCATGTGGAATCCGACCCCGTCAGGATATGTGGTTCTGGTAGGA
GACGAGAACCTAAAACAGTTCCCGCCTCCGTCTGAATTTTTGCTTTCGGTTTGGAAACCGAAGCCGCGCTCTT
GTCTGCTGCAGCGCTGCAGCATCGTTCTGTGTTGTCTCTGTCTGACTGTGTTTCTGTATTTGTCTGAAAATTA
20 GGGCCAGACTGTTACCACTCCCTTAAGTTTGACCTTAGGTCACTGGAAAGATGTCGAGCGGATCGCTCACAAC
CAGTCGGTAGATGTCAAGAAGAGACGTTGGGTACCTTCTGCTCTGCAGAATGGCCAACCTTTAACGTCGGAT
GGCCGCGAGACGGCACCTTTAACCGAGACCTCATACCCAGGTTAAGATCAAGGTCTTTTACCTGGCCCCGA
TGGACACCCAGACCAGGTCCCCTACATCGTGACCTGGGAAGCCTTGGCTTTTGACCCCCCTCCCTGGGTCAAG
CCCTTTGTACACCCTAAGCCTCCGCTCCTCTCTCCATCCGCCCGTCTCTCCCCCTTGAACCTCCTCGTT
25 CGACCCCGCCTCGATCCTCCCTTTATCCAGCCCTCACTCCTTCTCTAGGCGCCGGAATTCTGTTAACTCGAGGA
TCTAACCTAGGTCTCGAGTGTTTAAACACTGGGCTTGTGAGACAGAGAAGACTCTTGCGTTTCTGATAGGCA
CCTATTGGTCTTACTGACATCCACTTTGCCTTCTCTCCACAGGTGAGGCCTAGGCTTTTGCAAAAAGCTTGG
GCTGCAGGTGAGGCGGATCTGATCAAGAGACAGGATGAGGATCGTTTTCGATGATTGAACAAGATGGATTGC
ACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCCGGCTATGACTGGGCACAACAGACAATCGGCTGCTC
TGATGCCGCCGTTTCCGGCTGTGACGCGAGGGGCGCCCGGTTCTTTTGTCAAGACCGACCTGTCCGGTGCC
30 TGAATGAATGACAGGACGAGGACGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGC
TCGACGTTGTCACTGAAGCGGGGAGGACTGGCTGCTATTGGGCGAAGTGCCGGGCGAGGATCTCTGTCTATC
TCACCTTGCTCCTGCGGAGAAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCT
ACCTGCCCATTGACACCACAAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCG
ATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAAGTTTCGCCAGGCTCAAGGCGCGCAT
35 GCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGC
TTTTCTGGATTTCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTG
ATATTGCTGAAGAGCTTGGCGGCAATGGGCTGACCGCTTCTCGTGTCTTACGGTATCGCCGCTCCCGATT
GCAGCGCATCGCTTCTATCGCTTCTTGACGAGTCTTCTGAGCGGACTCTGGGGTTCGATAAAATAAAG
ATTTTATTTAGTCTCCAGAAAAAGGGGGAATGAAAGACCCACCTGTAGGTTTGGCAAGCTAGCTTAAGTAA
40 CGCCATTTTGCAAGGCATGGAATAACATAACTGAGAATAGAGAAGTTCAGATCAAGGTGAGGAACAGATGG
AACAGCTGAATATGGGCCAAACAGGATATCTGTGGTAAGCAGTTCTGCCCCGGCTCAGGGCCAAGAACAGAT
GGAACAGCTGAATATGGGCCAAACAGGATATCTGTGGTAAGCAGTTCTGCCCCGGCTCAGGGCCAAGAACAG
ATGGTCCCAGATGCGGTCCAGCCCTCAGCAGTTTCTAGAGAACCATCAGATGTTTCCAGGGTGCCCCAAGGA
CCTGAAATGACCCTGTGCCTTATTTGAACTAACCAATCAGTTCGCTTCTCGCTTCTGTTTCGCGCGCTTCTGCT
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55 See Figure 16